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## CLPXP FUNCTIONS IN CAULOBACTER AS A UNIVERSAL AND SPECIES-SPECIFIC PROTEASE

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**CLPXP FUNCTIONS IN *CAULOBACTER* AS A UNIVERSAL AND SPECIES-SPECIFIC  
PROTEASE**

A Dissertation Presented

by

ROBERT H. VASS

Submitted to the Graduate School of the  
University of Massachusetts Amherst in partial fulfillment  
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 2018

Molecular and Cellular Biology

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CLPXP FUNCTIONS IN *CAULOBACTER* AS A UNIVERSAL AND SPECIES-SPECIFIC  
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A Dissertation Presented

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ROBERT H VASS

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## **DEDICATION**

I dedicate this work to my father, mother, and siblings for the constant love and support necessary for traversing the doctoral path. This journey's sweetness centers on the unspoken, shared success coming from their support of my scientific research.

I also dedicate this dissertation to Ms. Rita Muskauski. Your vision challenged me to be matchless, hungry, and uncompromising. Thank you for igniting the pilot light.

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## ABSTRACT

### CLPXP FUNCTIONS IN *CAULOBACTER* AS A UNIVERSAL AND SPECIES-SPECIFIC PROTEASE

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Proteolysis shapes many aspects of cellular survival, including protein quality control and cellular signaling. Powered proteolysis couples ATP hydrolysis with a degradation force that actively probes and interrogates the protein population. ClpXP, exemplifies a conserved two-part protease system charged with powered proteolysis. This protease exists as a regulatory element (ClpX), and a compartmentalized, self-contained peptidase element (ClpP). In *Caulobacter crescentus*, ClpXP degradation plays a crucial role maintaining proteins that exhibit proper activity, and also triggers the start of cellular differentiation. Substrate elimination requires shared aspects of the protease from both quality control and precision protein destruction functions. Here, the regulatory element and peptidase must interact and recognize substrates for complete degradation. Discrimination between active function in quality control and protein removal used for signaling, challenges the protease to prioritize all encountered substrates. ClpXP accomplishes this task by fully exploiting ClpX N-domain specificity and a host of adaptors that effectively categorize protein degradation as quality control, cell-cycle dependent, or under replication fitness. Interestingly, ClpXP also functions to partially degrade some targeted protein substrates. One such constantly recognized protein that requires the ClpX N-domain, DnaX, undergoes partial proteolysis that generates two smaller protein forms. Multiple DnaX forms allow for clamp loading diversity, the isoforms produced in *Caulobacter* alternately phenocopy the activity of ribosomal slippage found in *E. coli*. The degradatory effects that ClpXP imposes within the cell best describe the proteases' function. Part of this process reveals some substrate recognition by a common mechanism, while a more elaborate delivery system coordinates recognition of other substrates. Further contrast of ClpX and ClpP activity between *Caulobacter* and *Escherichia* reveals that despite retaining universal purpose, these proteins evolved functions to meet the specific demands of their respective systems.



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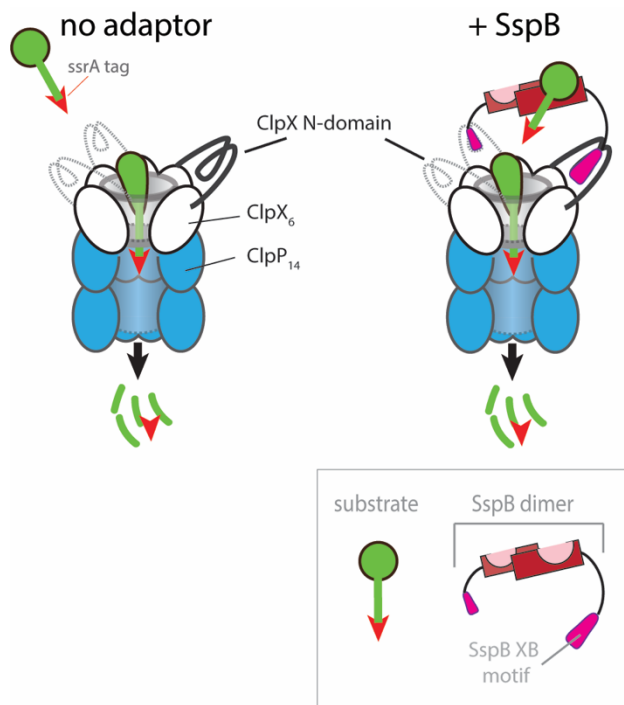
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complexed as a protease [2,3]. It is unknown however, if ClpX is always complexed with ClpP or if every successful protein-threading event by ClpX results in the destruction after translocation. This dissertation focuses on the ClpX efforts which remain within the protease partnership of ClpXP. Degradation is a definitive change to the proteome that affects many cellular processes. For example, ClpXP degrades its substrates into smaller peptides (~10-15aa in length) that remove the originally targeted protein's activity. The resulting small peptides are further degraded by other non-specific proteases into even smaller amino acid polymers that can be recycled or directly further the propagation of other cellular signals [4,5]. ClpXP performs conserved activity as a protease in a wide variety of organisms, suggesting its functional preservation across species. ClpX is not identical between species, yet known functions must remain conserved to promote ClpXP activity. A priori, activity of ClpX is dependent on the unfoldase to function similarly, and is shared between these highly conserved elements found between species. For example, the ATPase domain of ClpX binds and hydrolyzes ATP, providing the translocation power into the central pore, an action stabilized in combination with conserved pore loops that supply constant substrate grip [6]. ClpX possesses an additional, unique N-domain feature that varies between species, and a requirement for scaffolding adaptors or facilitating the direct recognition of some substrates [7]. The result of trans-translation is a good example of direct and adaptor mediated delivery exhibited through SsrA-tagged substrate degradation. Trans-translation rescues stalled ribosomes by adding an SsrA-tag on the carboxy-terminus, where ClpXP removes these incomplete translation products by proteolysis [8-10]. Degradation of these SsrA-tagged substrates happens independently of the ClpX N-domain [10]. However, recognition of substrates with the SsrA tag is enhanced by SspB (Figure 1.2), an adaptor that requires ClpX N-domain scaffolding [11,12]. In a sense, the adaptor and ClpX N-domain localizes substrate, but pore recognition ultimately dictates substrate recognition and removal [10]. Degradation occurs upon substrate translocation into the ClpP chamber, and ClpX must interact effectively with ClpP for proteolytic activity [13,14]. These aspects of ClpXP activity are conserved amongst





**Figure 0.2 Adaptors enhance substrate degradation but require the ClpX N-domain.**

organisms, and are also preserved between these various homologs [2,13,15-21]. Ultimately, this ensures similar mechanisms by which ClpX oligomerizes, binds ATP, and interacts with ClpP.

The combination of these independent, self and substrate interactions allows for both the variable and specific recognition by ClpX. Tuning these interactions actively shapes the ClpXP proteome, and makes sure the protease correctly degrades proteins by specifically

targeting substrates for irreversible destruction [6]. ClpX accomplishes this task through a network of degradation tags and adaptor proteins that alter substrate pools in an N-domain dependent manner [13,17-19,21] which influence protein homeostasis and cell growth [10,22-24].

## 1.2 Substrate recognition and degradation

ClpX is not essential to all organisms that contain this gene. This is the case for *E. coli*, where the protease is dispensable [3,23]. In contrast, *Caulobacter crescentus* requires ClpXP activity for survival, where the protease constantly removes the  $\beta$ -clamp binding toxin SocB, a protein substrate not found in *E. coli* [25]. In *Caulobacter*, the *clpX* promoter is independently induced from its cognate peptidase *clpP* promoter, allowing for independent, promoter-based change between these proteins. Selective expression effectively decouples the heat shock response (quality control) from required protein level changes originating from cell cycle progression [3], suggesting concentration drives proteolytic activity. However, these protease

levels do not drastically change, but the activity of the protease does, such as triggering the start of replication and differentiation in *Caulobacter*. Substrate degradation by ClpXP drives the transition from a motile cell type to a non-motile or “stalked,” replication-competent cell type [26-28]. Interestingly, ClpX induction corresponds to temporal based cues during differentiation, and presumed to sufficiently meet proteolytic demands required for this transition [22,23,29-31]. ClpXP must also fulfill regulatory roles when parallel processes exist, such as providing cell signaling and quality control, even when competition or simultaneous inputs are received. This is the job of the unfoldase, ClpX, the regulatory piece of the protease complex that recognizes and unfolds substrates into ClpP. ClpX is charged with sorting through and prioritizing efforts that may be solely dependent on its variable regions. These variable regions offer contacts that define separate ClpX N-domain activity from pore interactions, such as adaptor binding [7,12,27,29,32]. ClpX activity directly relates to the ability of discriminating between substrates, as proteolytic changes are driven by ability the recognition and translocate targets into the ClpP peptidase for destruction [16-18,20,21,33].

### **1.2.1 Coordinating degradation by recognition**

ClpXP targeted degradation occurs in several ways, where protein destruction comes from specific interactions. Substrates can be recognized by the unfoldase pore, selectively by N-domain, or a combination of both the pore and N-domain, or through adaptor proteins that mediate these transactions. Recognition represents a crucial first step in regulated proteolysis, critical for degradation where initial substrate engagement leads to subsequent translocation and degradation. Direct interaction by the ClpX pore may be the simplest and most conserved form of recognition that positively influences protein disassembly. Disassembly depends on recognition, as mutating the recognition sequence (or degron) discourages interactivity, which also negatively impacts degradation [34]. In this sense, active tethering of substrate with suppressed degron activity is insufficient for delivery, suggesting recognition alone is the critical step [35].

In addition to pore recognition, both *Caulobacter crescentus* and *E. coli* ClpX contains an N-terminal domain (N-domain) that provides additional binding surfaces for protein interactions. These N-domains can interact directly with the substrate through “enhancer tags” that promote self-delivery or provide extra real-estate for adaptor binding. The N-domain provides a platform for adaptors that scaffolds additional interaction [12] or expands contact to the unfoldase [35] that effectively orients substrates for efficient delivery and degradation. These N-domains further expand ClpX substrate pools to include otherwise normally unrecognized proteins [12,27,32,36]. *Caulobacter* utilizes these changes in targeted proteolysis to drive the initiation of cell cycle differentiation through layers of adaptor composition and assembly, effectively coupling protease activity and timing (reviewed in [27]).

### **1.2.2 Translocation into the proteolytic chamber**

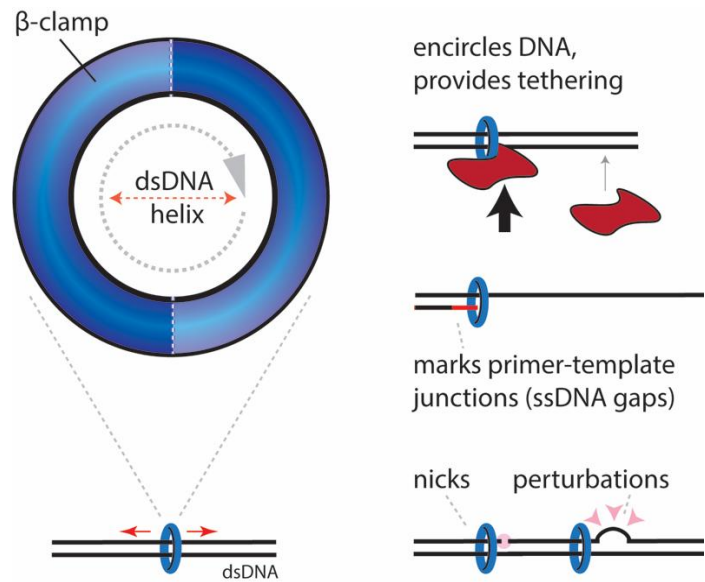
After substrate engagement, ClpX continuously threads substrate into the proteolytic chamber until degradation is complete [1]. ClpXP processively degrades substrates fully through successive translocation. The ability of the unfoldase to maintain substrate contact and peptidase interactions determines success. After recognition, substrates threaded through the unfoldase maintain attachment through constant pore loop contact, preventing early release by providing constant grip. These critical loop contacts ensure successive unfolding events that maintain translocation and perpetuate degradation [17-20] in an ATP dependent manner [6,16].

The force by ATP hydrolysis provides sufficient unfolding power to disassemble protein complexes [36] and unfold domains containing secondary structure [1,37]. Passage of proteins through the pore occurs in either an N- or C-terminal direction [38,39]. Additionally, the central pore of ClpX expands to accommodate several polypeptides, therefore allowing simultaneous translocation and degradation of multiple substrate targets (speculated in figure 8 of [40], shown

in [35]). These data suggest ClpX-ClpP-substrate interactions withstand translocation force while simultaneously maintaining degradation of multiple targets.

### 1.3 Sliding clamp use during DNA metabolism

Bacterial sliding clamps consist of 2 identical subunits in a head-to-tail orientation that forms a torus-shaped complex, where the inner cavity is large enough to encircle yet freely slide on DNA [41-43] (Figure 1.3). DNA metabolism utilizes sliding clamps to tether proteins onto DNA, thus maximizing their efficiency [41]. Tethering of the polymerase to the



**Figure 0.3 Sliding clamp architecture allows for its mobility, and facilitates DNA interactions.**

sliding clamp increases both the speed of nucleotide synthesis and lifetime of polymerase-DNA interaction to promote processivity. In spite of replication's semi-discontinuous nature, replication speed must meet the demands of completing genome duplication, especially during Okazaki fragment synthesis on the lagging strand. Here, clamp-based processivity ensures nucleotide incorporation on the leading strand does not outpace that of the lagging strand [44,45]. Sliding clamps also function as indicators that demark the interface of the primer-template junctions, seen as nicked or protuberances in DNA, or the sectional start of single-stranded DNA gaps. In this capacity, clamps correctly position proteins at locales that need attention, and ensure partner-proteins stably contact DNA long enough to complete their function [42,46]. Sliding clamps lack the ability to independently attach or fall off DNA. Since clamps do not spontaneously encircle or dissociate from DNA, chaperones tasked with clamp placement and

removal (called loaders) determine their function and activity. Loaders direct clamp usage by combining a location with the necessary interaction and mechanical force required for placement and removal [47].

#### 1.4 The DnaX role in the bacterial clamp loader

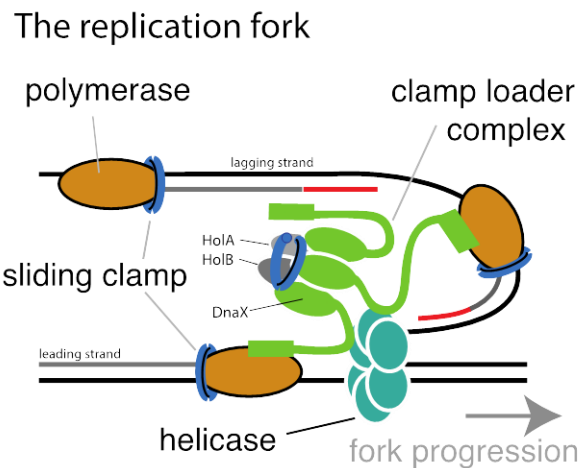
Clamp loaders are working complexes that engage, open, and distribute sliding clamps. These powered machines harness ATP-fueled activity, making passive contacts that change interactions between clamps and other proteins.

More specifically, ATP hydrolysis determines the loader-clamp interaction and conformation that drives placement and removal. In the well-characterized model organism *E.*

*coli*, the core clamp loader complex consists of five proteins; three DnaX proteins, and one each of HolA and HolB, for a ratio of 3:1:1 (DnaX:HolA:HolB). Only the DnaX subunits bind and hydrolyze ATP in the core loader, and along with HolAB in the core, provide contacts that hold the loader together (referenced in [48-50]).

The ATP bound state of DnaX

determines loader affinity for clamp, where hydrolysis changes conformational shape. ATP hydrolysis generates the required mechanical force that adjusts the loader/clamp interface while also increases clamp affinity, a process that effectively wrenches the clamp open (for reference [51]). Since only DnaX exhibits ATPase activity and coordinates the composition of the loader complex, [50], DnaX regulates clamp activity and placement.



**Figure 0.4 Loader coordinates replication effort through coupling organization and activity.**

Much in the same way the AAA+ protease ClpXP orchestrates targeted proteolysis in *Caulobacter*, replication effort and activity of sliding clamp is coordinated by DnaX. DnaX in *Caulobacter* must coordinate active clamp loading through passive interaction by its C-terminal tethering domain. Successful tethering by the DnaX C-terminal domain guides clamp use to target clamp-loading efforts. We were surprised that DnaX was targeted to ClpP in *Caulobacter*, and turned out to be a ClpX substrate that was not fully degraded. Instead, both *in vitro* and *in vivo* ClpXP exposure generated two shorter, stable, processed DnaX fragments. Comparatively, *E. coli* DnaX exists as two forms, a full-length version which is capable of utilizing all the contacts that are supplied by the gene, and a dispensable shorter version that results from translational frameshifting that is absent of any C-terminal tethering domains. The idea of separate organisms retaining altered versions of the DnaX protein was interesting, but gained traction as a strong conservation-based speculation as it became clearer these shorter fragments were generated through alternate pathways. Prior to our work, the understanding and activity for this short DnaX activity was based on *E. coli* studies that did not attribute unique activity to this fragment. So why was this fragment formed, yet also generated in other organisms? The literature largely speculated based on physical differences between the full-length and short forms, suggesting unproven ideas, independent versions, or activities of a bacterial clamp loader that was ultimately non-essential [52]. The effect these alternate forms of DnaX have would not be shown until several years later, seen facilitating increased survival after DNA damage [53,54] Regardless, combinations of these DnaX forms manifest at the replication fork, where the loader complex provides clamps while still maintaining contact with replication proteins helicase and polymerase [55,56]. Since proteolysis shapes many developmental transitions and responses in *Caulobacter*, it is revealing that ClpXP potentially impacts sliding clamp presence and availability during replication fork elongation. This imparts ClpXP with the power to successfully orchestrate loader efforts through targeted partial degradation, an activity that directly and positively influences DNA maintenance and metabolism.

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## **2. PROTEASE REGULATION AND CAPACITY DURING CAULOBACTER GROWTH**

*as written by Vass R.H., Zeinert R., and Chien P.,  
Current Opinion in Microbiology 2016*

### **General Chapter Overview**

This chapter highlights the regulation of powered proteolysis, necessary adaptations required for maintaining protein homeostasis, and activity changes that drive or respond to metabolic cues. *Caulobacter* employs many different types of proteases that shape its proteome, however, utilizes diverse methods for regulating these proteases. Regulation of these proteases has differing effects on the system by triggering events, balancing or managing interactions, and adjusting to fluctuations in the target substrate load. Growth requires the proper degradation response to a normal flux in activity, yet must also be carefully timed, maintained, and balanced even when these systems are challenged. Chapter 2 outlines how proteolysis drives cell-cycle differentiation in *Caulobacter*, maintains a replication balance, and handles an overflow of misfolded protein.

My contribution to this work is centered around the second section regarding how ClpXP balances DNA metabolism. I wrote this section, generated figure 2.2, and provided insight on the other sections. I consider myself lucky to be afforded the opportunity to incorporate some analysis and intuition into understanding how some of these protease interactions shape robust *Caulobacter* survival.

## 2.1 Abstract

Cell growth requires the removal of proteins that are unwanted or toxic. In bacteria, AAA+ proteases like the Clp family and Lon selectively destroy proteins defined by intrinsic specificity or adaptors. *Caulobacter crescentus* is a gram-negative bacterium that undergoes an obligate developmental transition every cell division cycle. Here we highlight recent work that reveals how a hierarchy of adaptors targets the degradation of key proteins at specific times during this cell cycle, integrating protein destruction with other cues. We describe recent insight into how *Caulobacter* manages DNA replication and repair through Lon and Clp proteases. Because proteases must manage a broad substrate repertoire there must be methods to compensate for protease saturation and we discuss these scenarios.

## 2.2 Highlights

- \* An adaptor hierarchy controls staged protein degradation during cell cycle progression.
- \* ClpXP balances dynamics of replication sliding clamps by proteolysis of both inhibitory and activating factors.
- \* The Lon protease accommodates a wide range of substrates independent of sequence, but also recognizes some specific targets based on sequence.
- \* Saturation of proteases must be managed by additional regulation or compensation.

## 2.3 Introduction

The regulated destruction of proteins is crucial for bacterial growth and development during normal and stress conditions. In the bacterium *Caulobacter crescentus*, regulated degradation of key proteins drives the cell cycle and depletion of replication factors during stress responses allows cells time to recover from these damages. In this review, we will give an overview of protein degradation in *Caulobacter* focusing on recent studies showing how regulated proteolysis by the Clp and Lon family of proteases impacts both normal and stress related growth. The common and unique substrate profiles of these proteases allow them to robustly provide for normal growth and respond to stress. Comparison of several different bacterial systems allows us to determine common themes reflecting broad responses to stress.

## 2.4 Energy dependent proteases in *Caulobacter*

Like most bacteria, regulated proteolysis in *Caulobacter* is accomplished by several energy dependent proteases. Although they differ in specific protein subunits, these ring-shaped proteases generally function by recognizing targets, then unfolding them using energy captured from ATP hydrolysis, ultimately threading these polypeptides into a chamber harboring active sites for peptide bond hydrolysis. Because of their design, these chambered peptidases cannot normally degrade folded or full-length polypeptides on their own and are solely dependent upon the active delivery of the target. The responsibility of target recognition falls on ATP-dependent chaperones that are encoded on separate proteins (such as the case with ClpXP) or domains (such as the case for the Lon protease) from the peptidase. (Figure 2.6.1A).

With important exceptions, these proteases completely and processively degrade their targets once engaged without obvious sequence preference [1,2]. Therefore the initial specificity of these proteases is the major determinant of how they will impact the cell. Protease target recognition arises from a combination of the intrinsic specificity of the protease and the use of

adaptor proteins to further tune proteolytic range [2]. Because protein degradation is irreversible, understanding this initial step of recognition is crucial.

## **2.5 Regulated proteolysis during the cell cycle**

In *Caulobacter*, regulated protein degradation during the cell cycle drives replication and developmental transitions. The essential regulator CtrA controls transcription of many cell cycle genes and is also a replication inhibitor. Removal of CtrA activity through degradation or posttranslational changes is therefore necessary so that cells can initiate replication during the G1-S transition. Genetic and cell biology studies during the last ten years revealed that degradation of CtrA requires the ClpXP protease, the auxiliary factors CpdR, RcdA and PopA, and the second messenger cyclic di-GMP [3-7]. Interestingly, these factors were not solely dedicated to CtrA degradation. For example, proteolysis of the chemotaxis protein McpA and the cyclic di-GMP phosphodiesterase PdeA during the G1-S transition required CpdR and ClpXP but not RcdA or PopA [5,8]. How these inputs collectively resulted in degradation of specific substrates at specific times was an outstanding question.

The pacemaker of proteolytic control during cell cycle is the cyclic phosphorylation of the CpdR adaptor. CpdR is phosphorylated by the same kinase cascade responsible for CtrA phosphorylation [9], but the outcome of this posttranslational modification is opposite for the two proteins. Like canonical response regulators, phosphorylation of CtrA activates it as a transcription factor, while phosphorylation of CpdR inhibits its ability to stimulate the ClpXP protease [5]. Thus, activation of CtrA also results in its stabilization. Similarly, inactivation of CtrA through dephosphorylation also catalyzes CtrA destruction because the same pathway dephosphorylates and activates CpdR [5,9].

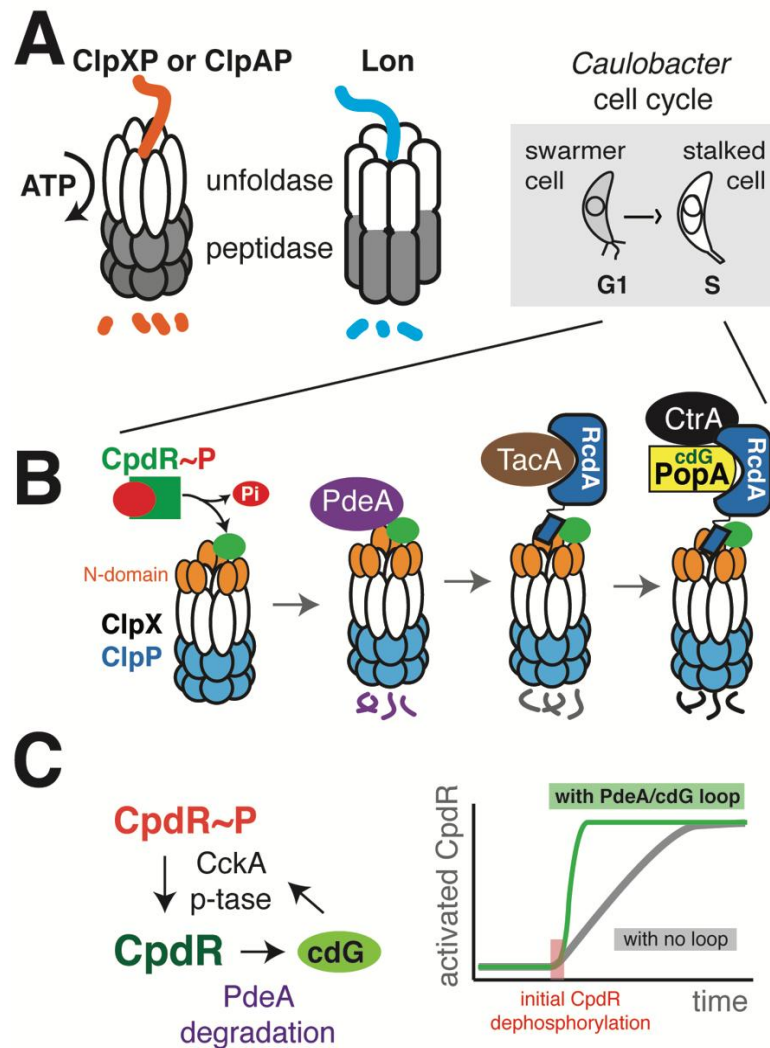
## 2.6 Adaptor hierarchies drive class specific substrate degradation

Recent biochemical work shows that CpdR, RcdA and PopA act as adaptors that hierarchically assemble to deliver substrates dependent on the degree of assembly [10-12] (Figure 2.6B). Reconstitution experiments using highly purified proteins showed that CpdR binds the ClpXP protease, priming it for recognition of substrates such as PdeA and McpA. Phosphorylation of CpdR causes it to release from ClpXP providing a simple mechanism for its control [11]. In addition to improved substrate recognition, CpdR-primed ClpXP could now bind RcdA, which was shown to bind several cargos [10]. RcdA could then deliver its bound target substrates, e.g. the developmental regulator TacA, to the CpdR-primed ClpXP [10]. Finally, RcdA also binds PopA and in the presence of cyclic di-GMP these proteins form a complex with CtrA [4,12]. Importantly, formation of this final complex promotes the robust degradation of CtrA by a CpdR-primed ClpXP, especially apparent in conditions where CtrA degradation by ClpXP alone is poor [10,12,13]. This model rationalized the importance of each of these proteins in the final degradation of CtrA as well as supported the need for cyclic di-GMP (Figure 2.6A).

Interestingly, it was recently shown that high levels of cyclic di-GMP causes dephosphorylation of CpdR/CtrA by switching the CckA kinase into a phosphatase [14]. As PdeA is a phosphodiesterase that limits cyclic di-GMP accumulation, it is tempting to speculate that degradation of PdeA upon dephosphorylation of CpdR can further stimulate CpdR activation by increasing levels of cyclic di-GMP. The advantage of this positive feedback is that activation of a subpopulation of CpdR would catalytically induce the conversion of the entire pool in short order, resulting in a sharper switch for proteolytic activation during the G1-S transition. These and other types of feedback regulation are likely needed for the robust transition between cell cycle stages crucial for normal development and growth (Figure 2.6C).

Although the specific example given above has been shown in *Caulobacter*, adaptor hierarchies are likely to be found in other bacterial systems. For example, during sporulation in





**Figure 0.6 Protein degradation by energy dependent proteases can be shaped by hierarchical adaptors.**

A. The Clp family of proteases are composed of unfoldases (ClpX or ClpA) paired with the ClpP peptidase. The Lon protease is a single polypeptide with these activities contained in different domains. B. The G1-S transition in *Caulobacter* is accompanied by morphological changes from a motile swarmer cell to a sessile stalked cell. At this transition, the dephosphorylation of CpdR initiates the assembly of an adaptor hierarchy that results in staged degradation of substrates. C. CpdR phosphorylation (and its activity) is ultimately controlled by CckA. CckA is a histidine kinase, but high levels of cdG cause it to switch to a phosphatase, resulting in increased dephosphorylation of CpdR. CpdR is directly responsible for delivering the cdG phosphodiesterase PdeA to the ClpXP protease. This sets up a putative positive feedback loop in which initial CpdR dephosphorylation can catalyze full conversion of the CpdR pool to an activated state.

*Bacillus subtilis*, degradation of the SpoIVA regulator by the ClpXP protease eliminates defective cells. This process requires the small protein CmpA that binds directly to ClpXP, but genetic evidence suggests the need for additional factors [15]. In this light, CmpA could be acting as part of an adaptor hierarchy that ensures only high quality spores endure. It also stands to reason that additional adaptor-dependent protease pathways will emerge that control sporulation, given the irreversible and critical nature of this developmental decision.

Finally, it is worth remarking that finding additional adaptor hierarchies is particularly challenging as protease adaptors are defined by their ability to stimulate substrate degradation by the protease. Yet biochemical validation of a protease substrate requires the prior knowledge of the adaptor in order to fully reconstitute this activity. Thus, addressing the circular challenge of novel adaptor/substrate identification is an outstanding question.

## **2.7 ClpXP balances critical aspects of DNA metabolism**

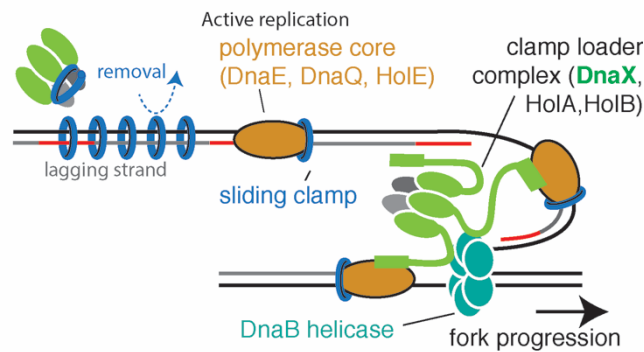
The ClpXP protease is essential in *Caulobacter*. The wide range of potential ClpP protease substrates leads one to assume that pleiotropic penalty paid by the loss of ClpXP would result in cell death [16]. However, a suppressor screen showed that a single toxin protein, SocB, was responsible for the truly essential nature of ClpXP [17]. The SocB toxin binds replication clamps and blocks replication elongation presumably by competing with DNA polymerase III for clamp [17] (Figure 2.7B). SocB activity is limited by the SocA antitoxin, which acts as an adaptor to deliver SocB to ClpXP. Like other adaptors, this activity requires the N-terminal domain of ClpX and the removal of the SocB toxin appears to be a major function of the ClpXP protease during normal growth conditions. SocB is upregulated in the presence of DNA damaging agents [17,18], suggesting that clamp inhibition may be an important aspect of the normal DNA damage response program. It is worth noting that although deletion of SocB toxin allows for strains to survive without ClpX, these cells have highly aberrant morphologies with dramatic reductions in

fitness and growth, consistent with a larger role for ClpX beyond the need for degrading this single toxin.

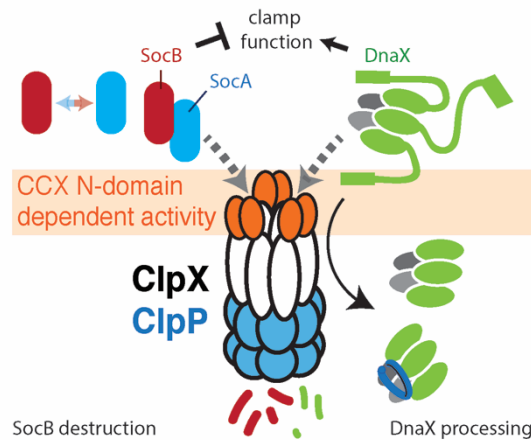
Another direct link between ClpXP and DNA metabolism in *Caulobacter* was identified in a proteomic approach that revealed the widespread nature of ClpP substrates [16]. DnaX is the ATP hydrolyzing core subunit of the clamp loader needed for the loading and unloading of the replication clamps (Figure 2.7A). DnaX was first identified in *E. coli*, where it was found to exist in two forms generated through programmed ribosomal frameshifting [19-21]. DnaX also exists as multiple forms in *Caulobacter* but these shorter forms are generated upon partial proteolysis by ClpXP [22]. Both forms are essential and strains engineered to express two DnaX variants locked in either long or short forms are viable. However, these strains are deficient in DNA damage tolerance, suggesting that dynamics of DnaX processing are important for this stress response [22]. Like the SocB example, processing of DnaX also requires the N-domain of ClpX (Figure 2.7B).

Recently, it was shown that the short form of DnaX is also important for DNA damage tolerance in *E. coli*, although it is dispensable for viability [23,24]. What is the short form doing? Prior work found that the short form is sufficient to load/unload clamps but lacks the regions needed to tether the full-length clamp loader to the replication fork [25] (Figure 2.7A). A tempting hypothesis is that the shorter DnaX clamp loader is dedicated to unloading [26], a feature that could be particularly useful during damaging conditions. Along these lines, a dedicated replication clamp unloader (Elg1) in yeast was recently described in yeast and shown to be important in DNA damage tolerance [27]. Another possibility is that the longer form of DnaX limits exchange of mutagenic polymerases due to the increased interactions with other pol III components [24]. In this manner, processing of DnaX by ClpXP may assist in polymerase exchange during damaging conditions.

A



B



**Figure 2.7 Replication clamp activity is controlled in part by the ClpXP protease.**

(A) DnaX is the protein in clamp loader that delivers the mechanical force needed for clamp opening. The full-length clamp loader is tethered to the replication fork by interactions between the C-terminus of DnaX, DNA helicase (DnaB) and the DnaE component of the polymerase. Removal of the C-terminus would release a shortened version of the clamp loader that could act as an unloader away from the replication fork. B. Under normal circumstances, both DnaX and SocB (with SocA acting as an adaptor) are degraded by ClpXP in an N-domain dependent manner. If the N-domain of ClpX is perturbed during damaging conditions (either by competition or direct damage) both SocB and full-length DnaX levels would rise providing compensating effects. Alternatively, if SocAB levels rise dramatically, this could itself compete for ClpXP, slowing the processing of DnaX and ensuring retention of clamp loading activity at the replication fork or damaged sites.

## 2.8 Integration of signals through the ClpX N-domain

From the above results, an intriguing speculation is that ClpXP may help balance clamp dynamics in *Caulobacter* by degrading an inhibitor of clamp and processing the clamp loader to generate an essential isoform (Figure 2.7B). Both these pathways rely on the unique N-domain of ClpX [17,22], a domain critical for adaptor binding [10,11,28]. If ClpXP activity is compromised due to direct damage or competition from other partners, protein levels of SocB would rapidly increase and DnaX would be less processed, resulting in more clamp inhibition and restricting clamp loader to the replication fork (Figure 2.7B). Such an occurrence could be beneficial during damaging conditions in order to prepare for quick restart of replication after repair and clamps were freed. Interestingly, direct damage to the ClpX N-domain has been suggested to underlie the transient stabilization of ClpXP substrates in *B. subtilis* during disulfide stress [29]. The use of a common protease for both loss of clamp function and activation of clamp activity would allow both these activities to change in concert if ClpXP is saturated by a surge in protease substrates. Thus, the N-domain of ClpXP would serve to integrate substrate load as an input with clamp dynamics as an output.

## 2.9 Lon degrades both folded and misfolded substrates

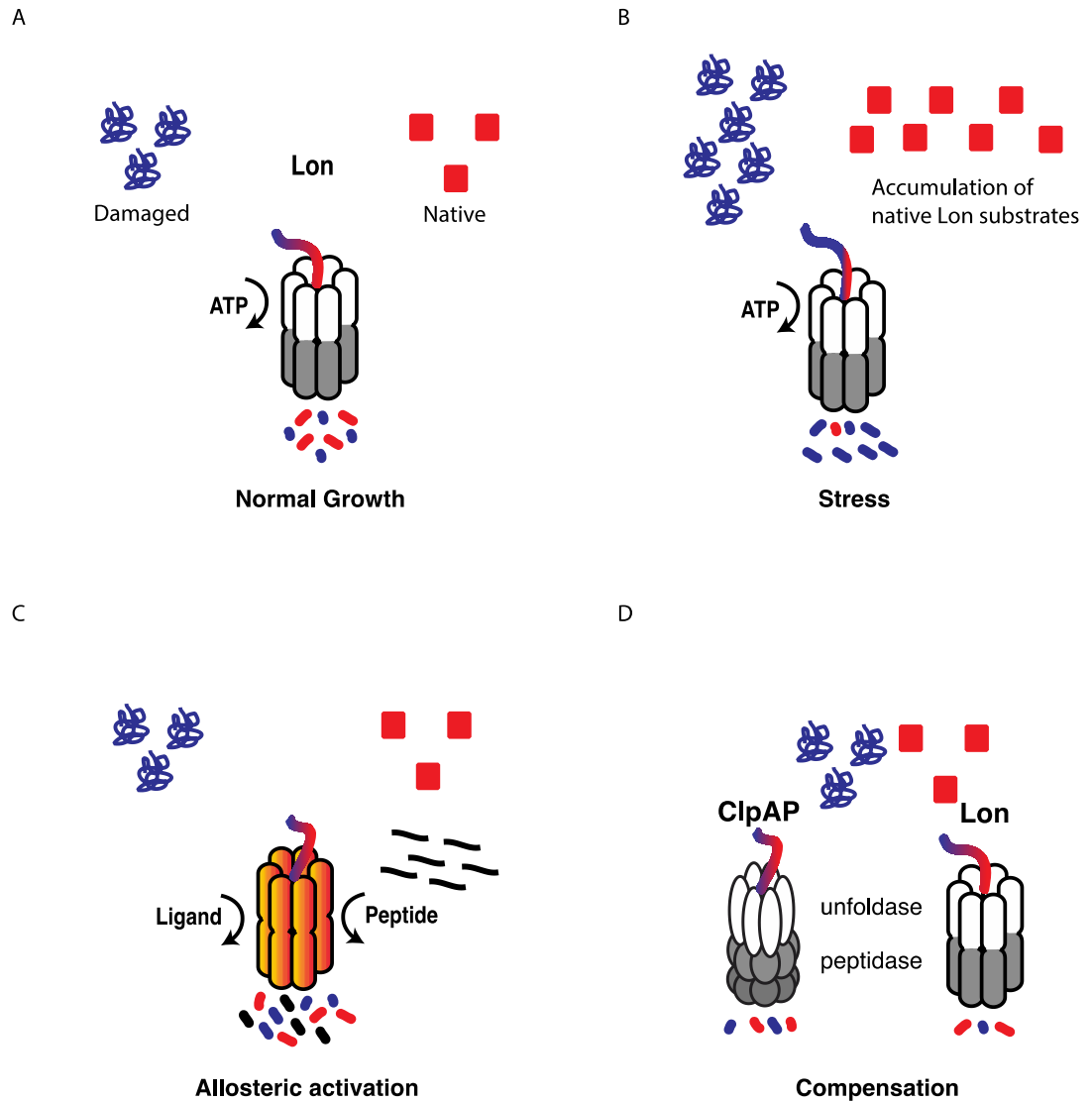
The Lon protease has long been known to be crucial for degrading misfolded or damaged proteins during stress conditions. In *Caulobacter*, Lon is responsible for both normal and stress related degradation of a several important regulators (Figure 2.9A). For example, the CcrM methylase is responsible for epigenetic regulation of a number of cell cycle genes [30] and its levels are partially managed by Lon-dependent proteolysis [31]. The SciP protein is a cofactor for CtrA that prevents activation of CtrA controlled genes during the G1 phase of the cell cycle [32,33]. Lon degradation of SciP during the cell cycle is important to remove SciP so that CtrA regulated genes can be activated [13]. Lon was recently shown to degrade the replication initiator

DnaA, a function particularly important during proteotoxic stress and starvation conditions [34,35].

The promiscuous nature of Lon has costs and benefits. Because any protein can misfold, a protease that eliminates misfolded proteins must have rather broad specificity. In fact, Lon is thought to recognize features of misfolded proteins such as exposed hydrophobic elements for most of its quality control targets, rather than specific sequences [36]. That said, Lon also clearly recognizes specific substrates even when they are folded, such as DnaA [34]. In addition, Lon specificity can be augmented by adaptors, as shown recently in *Bacillus subtilis* where adaptor-dependent Lon degradation controls cell motility upon surface contact [37]. Because Lon must recognize so many targets, it stands to reason that saturation of this protease might readily occur during damaging or stress conditions. Cases of protease saturation have been described and in some cases leveraged for synthetic biology [38-40]. Nonetheless, the unconstrained increase in substrates (either damaged or native) could lead to harm if left unchecked (Figure 2.9B).

## **2.10 Cellular responses to protease saturation**

How could protease systems respond to this toxic consequence? One way is to increase protease capacity through increased production or increased activity. In fact, allosteric stimulation of Lon has been demonstrated *in vitro* and *in vivo* where substrate recognition by Lon stimulates the degradation of a second substrate (Figure 2.9C) [34,41]. In *Caulobacter*, stimulation of Lon by misfolded protein substrates is thought to underlie the loss of DnaA that arrests the cell cycle during proteotoxic stress [34]. Another method to combat protein overflow is by upregulating other protease activities (Figure 2.9D). For example, like Lon, the ClpAP protease also degrades poorly folded substrates such as casein [42] and in *Caulobacter*, the ClpP family of proteases has been implicated in degradation of DnaA [43]. Consistent with this



**Figure 2.9 Lon balances normal degradation with stress related duties.**

A. Lon maintains homeostasis by balancing two main functions: Protein quality control and destruction of natively folded substrates. B. Stress results in the accumulation of native or damaged proteins that may saturate Lon and result in a subsequent buildup of other substrates. C. Allosteric activation of Lon by ligands or misfolded proteins can compensate for the extra protease demand in stress conditions. D. Saturation of Lon can also be compensated for through upregulation of other proteases, such as ClpAP, which has some overlapping specificity with Lon.

compensation model, overexpression of ClpAP protects against the toxic accumulation of DnaA in cells lacking Lon (unpublished; JL, PC). Given the widespread nature of ClpAP and Lon in gram-negative bacteria, perhaps similar compensation will be found elsewhere.

Finally, we note that Lon has long been known to be involved in DNA damage tolerance. Lon's ability to regulate DNA damage dates back to Evelyn Witkin's initial genetic studies of a UV sensitive, naturally Lon-deficient, B strain of *E. coli* [44]. In addition to radiation sensitivity, it was shown that Lon mutants also showed stabilization of  $\beta$ -galactosidase fragments leading to the discovery of suppressors of Lon (Sul)[45]. These suppressors were mapped to Sula, an FtsZ inhibitor upregulated in response to UV stress [46,47]. When Lon is absent Sula accumulates and irreversibly blocks cell division, ultimately resulting in cell death [48]. Whether Lon regulates DNA damage responses across bacteria is unclear, particularly in bacteria without obvious Sula homologs. In *Caulobacter crescentus* no Sula homolog exists. However, the small proteins SidA and DidA block cell division during DNA damage by inhibiting the divisome proteins FtsW and FtsN, respectively [18,49]. However, it remains to be seen if Lon even plays a role in the protein turnover of these cell division inhibitors.

## **2.11 Conclusions**

Regulated protein degradation is critical to all life. For AAA+ proteases such as ClpXP and Lon, ensuring specificity is crucial because degradation is irreversible. Substrate choice can be controlled by the intrinsic activity of the protease and/or tuned by adaptor proteins. In some cases, such as during the *Caulobacter* cell cycle, adaptor proteins assemble into hierarchies that deliver different substrate classes depending on their degree of assembly [10-12].

The ClpXP protease in *Caulobacter* seems to play a fundamental role in replication as it degrades both an inhibitor of replication clamps and generates an essential isoform of the clamp loader complex through partial proteolysis. The Lon protease degrades both misfolded proteins



of varying sequences and specific folded proteins, but must balance this breadth of substrate recognition with the cost of being readily saturated. Saturation of one protease type can be balanced by the increased protease activity of the same type (either through increased synthesis or stimulation) or by compensation through upregulation of another protease with overlapping specificity. In some cases, this saturation might result in additional regulation, such as when two opposing factors are recognized through the same protease. Understanding these increasingly diverse mechanisms of protease regulation and their impact on cell growth or stress response are a rich topic for future exploration.

## **2.12 Acknowledgements**

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### **3. IDENTIFICATION OF CLPP SUBSTRATES IN *CAULOBACTER CRESCENTUS* REVEALS A ROLE FOR REGULATED PROTEOLYSIS IN BACTERIAL DEVELOPMENT**

*as written by Bhat N., Vass R.H., Stoddard P.R., Shin D.K., and Chien P.,  
Molecular Microbiology 2013*

#### **General Chapter Overview**

This chapter highlights the results of a proteomic trapping survey, setting a course for further cellular and biochemical testing. The major contribution for which I am responsible for was performing the ClpP depletion in *Caulobacter* cells followed by the double tandem pull down to show the enrichment of substrates upon ClpP depletion. Minor contributions for this work include the material and methods section, performing the tandem affinity purification during ClpP and ClpP depletion conditions along with the design and construction of several protein and fusion protein expression vectors. Also generated and supplied for *in vitro* experiments were purified proteins and protease complex, ClpXP used for substrate validation through gel based and fluorescence based degradation assays.

Through characterization of the substrate list generated by this work, I netted the ClpXP client substrate DnaX amongst other substrates; which required validation for ClpP targeted degradation. The vast majority of my thesis work is based upon DnaX and the phenomenon of partial proteolysis that was established by protein characterization and derived in detail in the subsequent chapters of this dissertation.

### 3.1 Abstract

Energy dependent proteases ensure the timely removal of unwanted proteins in a highly selective fashion. In *Caulobacter crescentus*, protein degradation by the ClpXP protease is critical for cell cycle progression; however, only a handful of substrates are currently known. Here, we use a trapping approach to identify putative substrates of the ClpP associated proteases in *C. crescentus*. Biochemical validation of several of these targets reveals specific protease recognition motifs and suggests a need for ClpXP specific degradation beyond degradation of known cell cycle regulators. We focus on a particular instance of regulated proteolysis in *Caulobacter* by exploring the role of ClpXP in degrading the stalk synthesis transcription factor TacA. We show that TacA degradation is controlled during the cell cycle dependent on the ClpXP regulator CpdR and that stabilization of TacA increases degradation of another ClpXP substrate, CtrA, while restoring deficiencies associated with prolific CpdR activity. Together, our work reveals a number of new validated ClpXP substrates, clarifies rules of protease substrate selection, and demonstrates how regulated protein degradation is critical for *Caulobacter* development and cell cycle progression.



### 3.2 Introduction

The proteome is maintained by the synthesis and removal of cellular proteins at specific times and places in response to internal and external signals [1,2]. Proteolysis is a robust process that ensures the elimination of proteins when they are no longer needed; however, the irreversible nature of protein degradation demands stringent selectivity in substrate choice. In bacteria, regulated protein degradation is often governed by energy dependent proteases, also known as AAA+ proteases. One member of this family is ClpXP, an oligomeric machine formed from the ClpX unfoldase and ClpP peptidase. ClpP alone is incapable of degrading folded proteins and requires associated unfoldases, such as ClpX, that engage substrates via binding of short peptide sequences (recognition motifs). Targets are actively unfolded and translocated into the ClpP chamber where they are processed to peptides. The ClpP system has been best characterized in *E. coli* where the Clp family proteases regulate many pathways such as protein quality control, exit from stationary phase and response to damaging agents [1].

*Caulobacter crescentus* undergoes an obligate morphological differentiation from non-replicative swarmer cells to replication competent stalked cells every cell-cycle. This remodeling depends on proper coordination of regulatory proteolysis and synthesis. For example, ClpX and ClpP are essential in *Caulobacter* [3] and regulate proteins important for cell development, cell-cycle progression [4-7], chemotaxis [8,9], and replication [10,11]. Although several ClpXP substrates have been identified in *Caulobacter*, expression of mutant nondegradable alleles of these proteins or blocking degradation of these substrates does not mirror the many effects that arise when ClpXP is lost [4-6]. Thus the ClpXP dependent changes in development and cell cycle progression must rely on degradation of other as yet unknown substrates.

Here, we present a list of potential substrates of the ClpP protease system in

*Caulobacter* that includes known substrates, those documented from other bacteria, and previously unknown candidates. *In vitro* biochemical experiments validate a number of these targets and confirm the preservation of particular ClpXP recognition motifs across species. Next, we identify a role for the zinc-binding N-terminal domain of ClpX for *Caulobacter* viability and endogenous substrate selection. Finally, we focus on the stalked cell regulator TacA, a candidate substrate found in our trap. We show that TacA is proteolysed by ClpXP *in vitro* and that TacA is selectively degraded during cell-cycle progression dependent on the ClpXP regulator CpdR. Interestingly, stabilization of TacA also affects the degradation of CtrA, another CpdR dependent ClpXP substrate, suggesting a linkage between TacA, CpdR and CtrA. Finally, we find that prolific TacA degradation contributes to the stalkless phenotype of cells lacking the PleC phosphatase as stabilization of TacA partially rescues stalked cell formation in this mutant.

### 3.3 Results

#### 3.3.1 Construction of a ClpP trap in *Caulobacter crescentus*

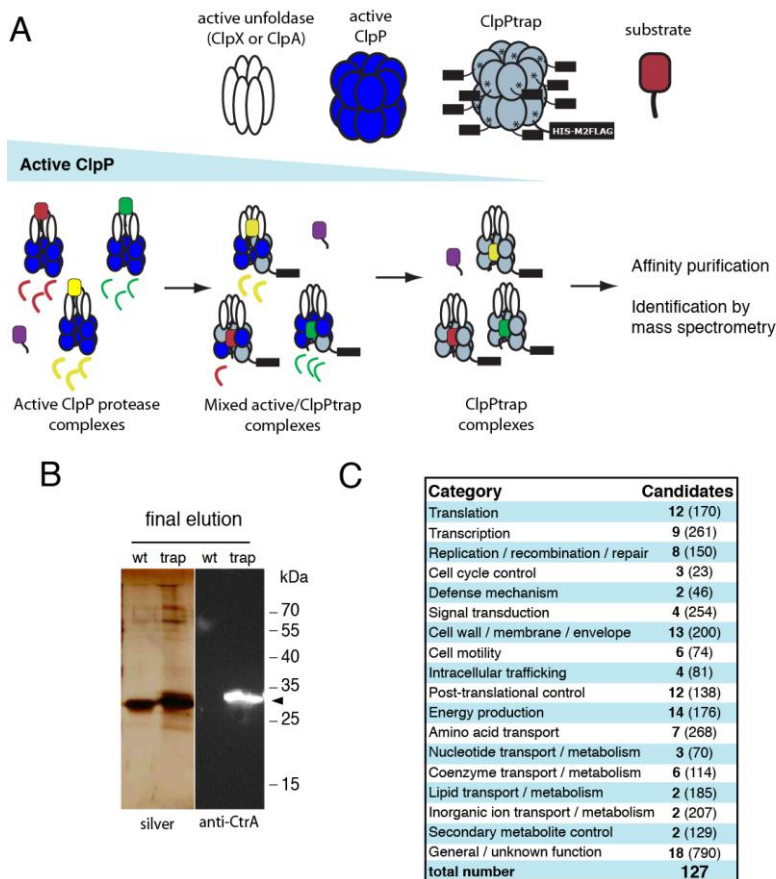
We identified novel substrates of ClpXP in *C. crescentus* using the strategy outlined in Figure 3.1 similar to that performed previously in *E. coli* [12]. Briefly, we mutated the active site serine of ClpP and appended a C-terminal tandem affinity tag to generate ClpPtrap (Figure S1<sup>\*</sup>). Substrate proteins are recognized by the native active ClpX or ClpA unfoldases and translocated into the inactive chamber of ClpPtrap [12-14]. The substrate-containing ClpPtrap complexes are then rapidly purified through successive rounds of affinity chromatography. Our initial efforts in wildtype cells were unsuccessful, likely due to incorporation of active subunits in the ClpPtrap oligomer; however, depletion of the chromosomally encoded ClpP while expressing ClpPtrap improved the recovery of candidates (Figure 3.1). Known substrates of ClpXP, such as the master regulator CtrA, were specifically enriched in the trapped substrate pool (Figure 3.1 and S1<sup>\*</sup>) and subsequent mass spectrometry identified 127 unique proteins in the eluted fraction

(Figure 3.1; Table S1<sup>\*</sup>). We pruned this list with high stringency requirements (see Supplemental Information) to generate a candidate list of 32 proteins (Table 3.1). We note that likely ClpP substrates (such as LexA and others shown below) fail to meet these more stringent requirements, but are clearly identified in our trap (Table S1<sup>\*</sup>); therefore, these lists should not be considered exhaustive, but reflect a high confidence subset of ClpP substrates. *Caulobacter* ClpP substrates (CtrA and SspB [3,6,15]) and Western blot analysis showed the capture of other known ClpXP substrates such as PdeA [16,17] that were not detected by mass spectrometry (Figure S1<sup>†</sup>). Interestingly, a number of the candidates found by mass spectrometry had been identified in other proteomic surveys as potential ClpP regulated targets. For example, several are annotated in *Caulobacter* as substrates of the trans-translation pathway [18], wherein polypeptides are cotranslationally appended with degradation tags that target them to either ClpXP or ClpAP. It is possible that some of these candidates are only targets of ClpP when tagged by the *ssrA* peptide. We also find conservation of ClpP substrates among widely divergent organisms as several candidates are known to be degraded in a ClpP dependent fashion in either *E. coli* or *B. subtilis* [12,19-21]. Finally, we identify a number of substrates previously shown to be unstable in *Caulobacter* whose regulation can now be assigned to ClpP [22,23].

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<sup>\*</sup>direct reference to Bhat et al., *Mol Microbiol.* 2013 Jun;88(6).

<sup>†</sup> direct reference to Bhat et al., *Mol Microbiol.* 2013 Jun;88(6).



**Figure 3.1 ClpP trapping strategy and substrate characterization.**

**A.** Inactive oligomers of ClpP can capture substrates delivered to them by active unfoldases. Depletion of endogenous, active ClpP enriches for inactive ClpPtrap oligomers. Affinity purification followed by mass spectrometry identifies candidate substrates. **B.** Substrates are co-purified with ClpPtrap, but are absent when an affinity tagged active ClpP is used. Western blotting with antibodies recognizing the known ClpXP substrate CtrA (black markers) confirms trapping procedure. Confirmation of additional substrates by Western blotting is shown in Figure S1\*. **C.** Candidate substrates are widely distributed across many functional categories as annotated by COG groups (NCBI), total numbers of proteins in each category shown in parentheses. The ClpP candidate pool shown in Tables 1 and S1\* is distributed across a wide range of functional categories. Mass spectrometry results identified two previously known ClpP dependent fashion in either *E. coli* or *B. subtilis* [12,19-21]. Finally, we identify a number of substrates previously shown to be unstable in *Caulobacter* whose regulation can now be assigned to ClpP [22,23].

\* direct reference to Bhat et al., Mol Microbiol. 2013 Jun;88(6).

### 3.3.2 Recognition motifs for ClpXP are preserved across species

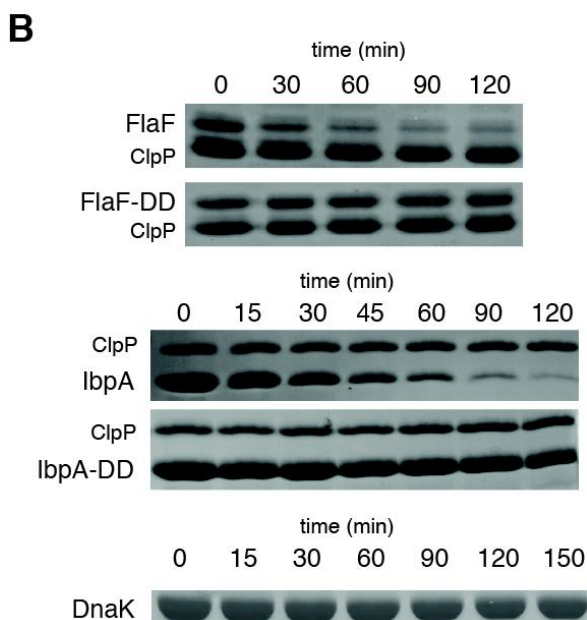
We next used a biochemical approach to validate putative ClpP substrates. We initially focused on candidates with known ClpXP recognition motifs classified by previous studies in *E. coli* by recombinantly expressing, purifying and biochemically validating several "C-terminal class I" family substrates (distinguished by the presence of Ala-Ala at the extreme C-terminus) [12]. Of the six candidates of this class that we tested, four (FlaF, IbpA, CtrA, CC2882) were recognized by ClpXP *in vitro* [15] (Figure 3.2 and S2\*). Furthermore, the ClpXP dependent degradation of CC2323, which contains SA rather than AA, agrees with the known tolerance of this motif (Figure S2A,B\*). Mutating the C-terminal residues of FlaF and IbpA to Asp-Asp eliminates recognition, revealing the importance of the C-terminal dipeptide for specific recognition by ClpXP (Figure 3.2A,B). The validated ClpXP substrates do not share any obvious common functional or sequence features that distinguish them from nondegraded substrates (DnaK and CC0321). Thus, our data show, as would be predicted, that while conserved protease recognition motifs can dictate targeting by a protease, the simple presence of a particular motif does not ensure that a protein will be directly recognized by a particular protease.

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\* direct reference to Bhat et al., *Mol Microbiol.* 2013 Jun;88(6).

**A**

substrate	C-Terminus	degraded by ClpXP?
CC2258 (IbpA)	EGEKAA	Y
CC1459 (FlaF)	GRADAA	Y
CC3035 (CtrA)	EQVNAA	Y
CC2882	AASVAA	Y
CC0321	FEATAA	N
CC0010 (DnaK)	DNKPAA	N
CC2323	RRAQSA	Y
CC0360	KRKRRR	Y
CC3315 (TacA)	DMKEAG	Y



**Figure 3.2 Trapped substrates reveal conserved motif requirements for ClpXP proteolysis.**

**A.** Select substrates were cloned, expressed recombinantly and purified, then assayed for *in vitro* degradation by ClpXP. Table lists CC annotation / gene name, sequence of the C-terminal six residues, and whether the candidate substrate was degraded by ClpXP in standard conditions (see Methods and Figure S2\*). **B.** IbpA and FlaF are both degraded by ClpXP and mutation of their C-terminal Ala-Ala motif eliminates degradation. DnaK is not degraded by ClpXP *in vitro*.

\* direct reference to Bhat et al., Mol Microbiol. 2013 Jun;88(6).

### 3.3.3 The N-terminal domain of ClpX is critical for its *in vivo* function

We identified CC0360, a putative ornithine decarboxylase, as a likely ClpP substrate using our most stringent requirements (Table 1) and found that it was rapidly degraded by ClpXP *in vitro* (Figure 3.3A). The lack of a C-terminal Ala-Ala motif in CC0360 (Figure 3.2A) prompted us to explore how ClpXP was recognizing this substrate. The N-terminal zinc-binding domain of ClpX is unique to the ClpX family of unfoldases and is required for recognition of some substrates, such as MuA, lambdaO and FtsZ for the *E. coli* ClpX [24-26]; but dispensible for degradation of others, such as CtrA and ssrA-tagged proteins for the *C. crescentus* ClpX (Figure 3.3A,B and Figure S2\*) [15,27]. As in *E. coli*, the N-terminal domain of the *C. crescentus* ClpX is also critical for adaptor binding, as seen in the case of SspB [27]. Interestingly, we found that CC0360 was exclusively degraded by the full-length ClpXP complex and not by a version of ClpX lacking the N-terminal domain ( $\Delta$ NClpXP; Figure 3.3A). A similar, but less pronounced effect was seen with CC2882 (Figure S2D<sup>†</sup>), while other substrates, such as FlaF was recognized by  $\Delta$ NClpXP with rates similar to full-length ClpXP (Figure S2D\*). Because ClpX is essential in *Caulobacter*, we tested the importance of the N-terminal domain of ClpX *in vivo* and found that  $\Delta$ NClpX could not support viability (Figure 3.3C). We interpret this to mean that at least one important role for the full-length ClpXP *in vivo* is the degradation of substrates which rely on the N-terminal domain of ClpX (such as CC0360). This, of course, does not rule out other crucial roles for the N-terminal domain of ClpX, such as adaptor binding or localization.

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\* direct reference to Bhat et al., *Mol Microbiol.* 2013 Jun;88(6).

† direct reference to Bhat et al., *Mol Microbiol.* 2013 Jun;88(6).

### 3.3.4 TacA is degraded in a regulated fashion by ClpXP during the cell cycle

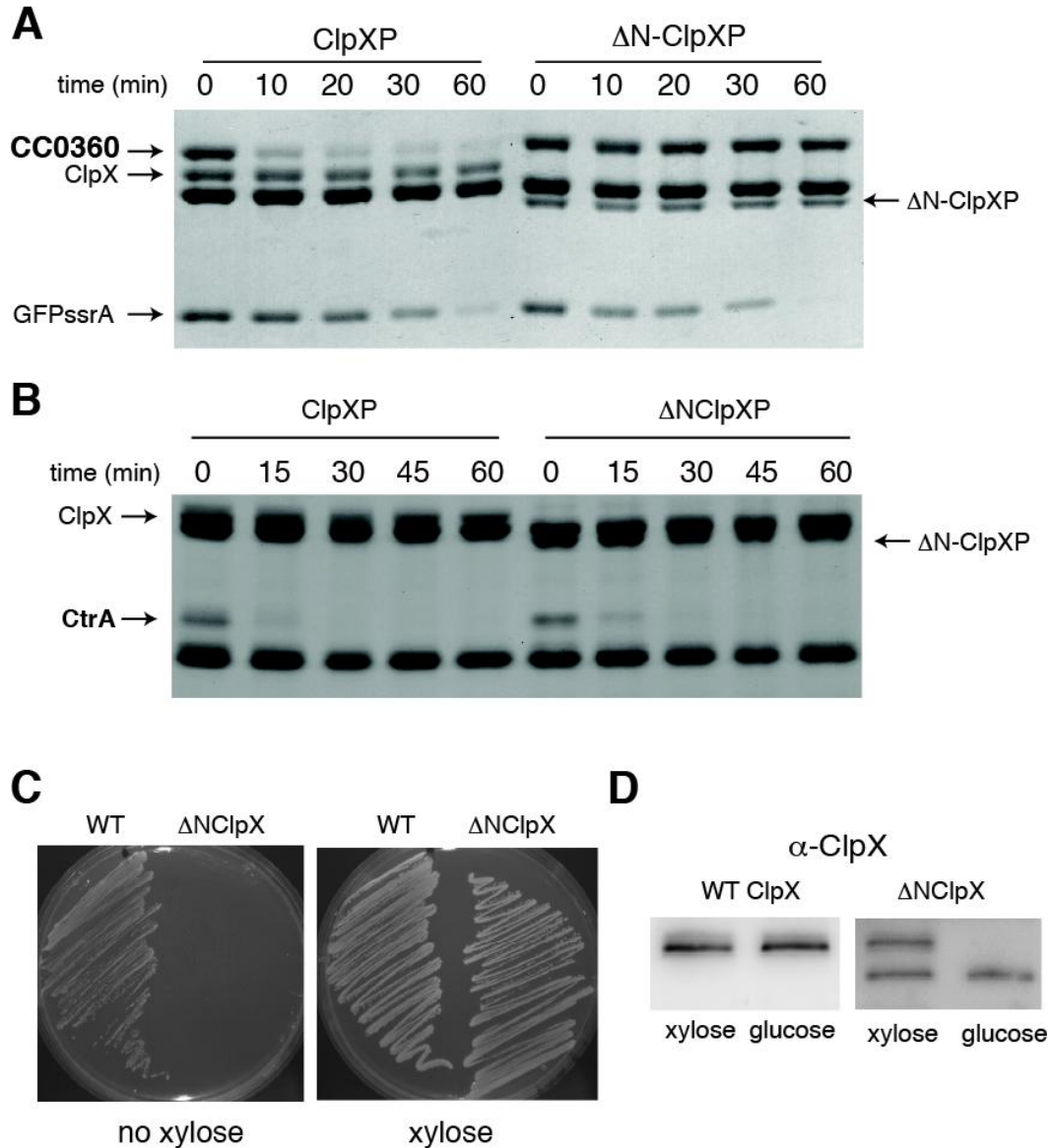
Stalk synthesis is a programmed developmental step in the life cycle of *Caulobacter* and is controlled transcriptionally by the  $\sigma^{54}$  dependent response regulator TacA [7,28,29]. TacA was identified in our trapping study and we found that the purified protein was degraded *in vitro* by ClpXP (Figure 3.4A). ClpXP recognizes the TacA via its C-terminal residues (ending in MKEAG-cooh) as mutation of the C-terminal dipeptide AG to DD prevents degradation (Figure 3.4A). An active, epitope-tagged variant of TacA [30] was degraded *in vivo* and stabilized by the same mutations that eliminate ClpXP recognition *in vitro* (Figure 3.4B and S4B<sup>\*</sup>). The regulator CpdR is required for the polar localization of ClpXP during the cell cycle [5] and is critical for the *in vivo* degradation of all known ClpXP substrates to date [5,7,16]. As predicted from these observations, and in accordance with its ability to be degraded by ClpXP *in vitro* (Figure 3.4A), we saw that the stability of TacA was dependent on CpdR *in vivo* (Figure 3.4B), further supporting its identification as a true ClpXP substrate. Stabilization of TacA results in smaller colonies when cells are inoculated into low-percentage agar media, suggesting that TacA degradation may impact normal growth, differentiation or motility (Figure SD,E<sup>\*</sup>).

A constitutively expressed M2-TacA was degraded at the G1-S transition in a CpdR-dependent fashion (Figure 3.4C and S4C<sup>\*</sup>), similar to that seen for CtrA [5,6]. Interestingly, cells expressing the nondegradable M2-TacA-DD show a delay in CtrA accumulation when compared to cells expressing M2-TacA (Figure 3.4C). Because CtrA positively regulates its own expression late in the cell cycle [31], this result suggests that stabilized TacA results in lower

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<sup>\*</sup> direct reference to Bhat et al., *Mol Microbiol.* 2013 Jun;88(6).





**Figure 3.3 The N-terminal domain of ClpX is a critical modulator of protease specificity.**

A. CC0360 degradation relies on the N-terminal domain of ClpX even though ssrA tagged substrates (GFP-ssrA) are degraded readily by both constructs. B. CtrA is recognized by both full length and ΔN-ClpX. C. The N-terminal domain of ClpX is essential for viability. Cells expressing a xylose inducible copy of *clpX* as the sole chromosomal copy and plasmids constitutively expressing either full length (WT) or ΔN-ClpX variants plated on inducing (xylose) or noninducing (no xylose) media. D. Western blot analysis using antibodies specific to *C. crescentus* ClpX confirm the constitutive expression of ΔN-ClpX from the plasmid in both inducing (xylose) and noninducing (glucose) conditions. Blots are registered so that upper bands (full length ClpX) are aligned.

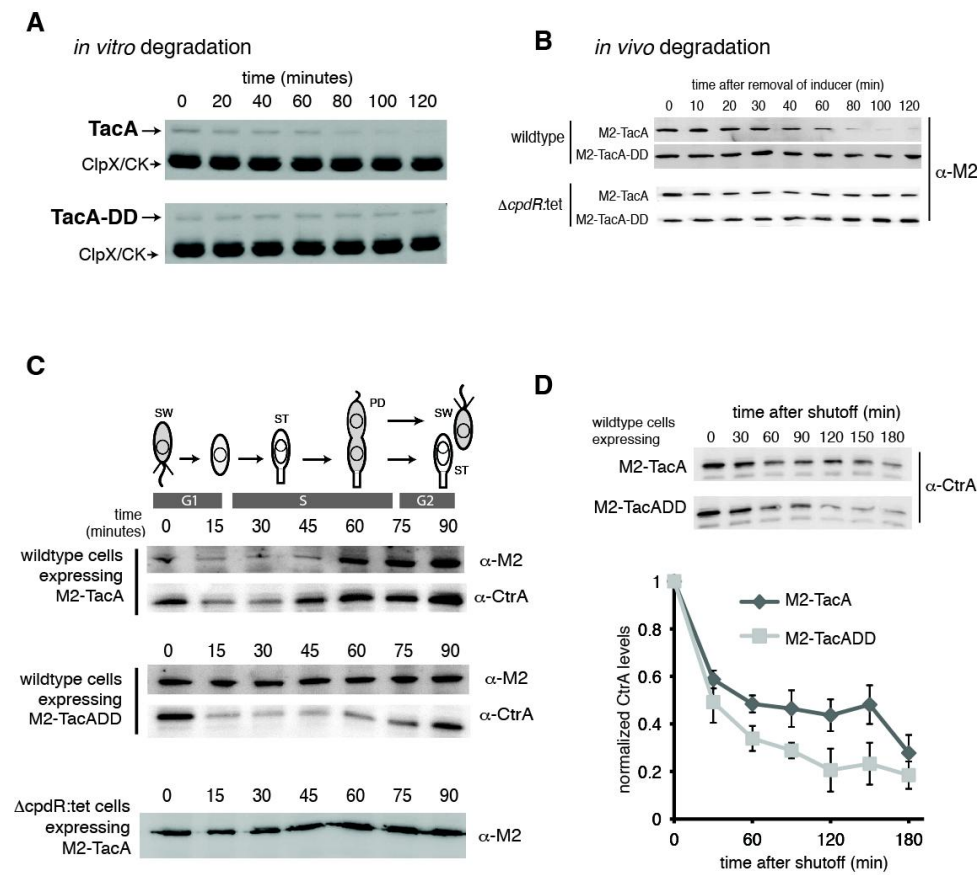
CtrA activity. Our data support a posttranslational role for this lower CtrA activity as CtrA is degraded more rapidly in cells expressing the nonproteolyzed M2-TacA-DD compared with cells expressing the properly degraded M2-TacA (Figure 3.4D).

### 3.3.5 Stabilization of TacA restores stalk formation in *pleC* mutants

Our results suggest proteolysis of TacA is integrated with changes in CpdR activity which in turn are dependent on the changing status of DivK phosphorylation during the cell cycle [32]. DivK phosphorylation by DivJ is countered by the PleC phosphatase and cells depleted of PleC have reduced CckA kinase activity resulting in lower levels of phosphorylated CpdR (depicted schematically in Figure 3.5A) [33]. Based on these observations, we hypothesized that the defects in stalk formation of *pleC* mutant cells [34] may arise in part from the rapid turnover of TacA driven by persistent dephosphorylation of CpdR. If our model is correct, then expression of a nondegradable TacA should restore stalk formation in  $\Delta pleC$  cells. We tested our hypothesis by replacing the endogenous allele of TacA in  $\Delta pleC$  cells with a nondegradable TacA-DD variant. Because stalked and swarmer cells are difficult to distinguish by size alone, we counted the number of predivisional cells in a mixed population with visible stalks (as illustrated in Figure 3.5B). Over 90% of wildtype predivisional cells had visible stalks, while 2-3% of predivisional  $\Delta pleC$  cells expressing normal TacA have stalks (Figure 3.5C). Expressing a stabilized variant of TacA in  $\Delta pleC$  cells increased the fraction of stalked predivisional cells 5-fold (Figure 3.5C). Consistent with these findings, stalk elongation in  $\Delta pleC$  during phosphate limitation is also increased when TacA is stabilized (Figure S5\*). Collectively, our results show how CpdR activity, which is normally regulated during cell cycle, can affect the developmental program of stalk formation via degradation of the novel ClpXP substrate TacA.

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\* direct reference to Bhat et al., *Mol Microbiol.* 2013 Jun;88(6).



**Figure 3.4 TacA is degraded by ClpXP.**

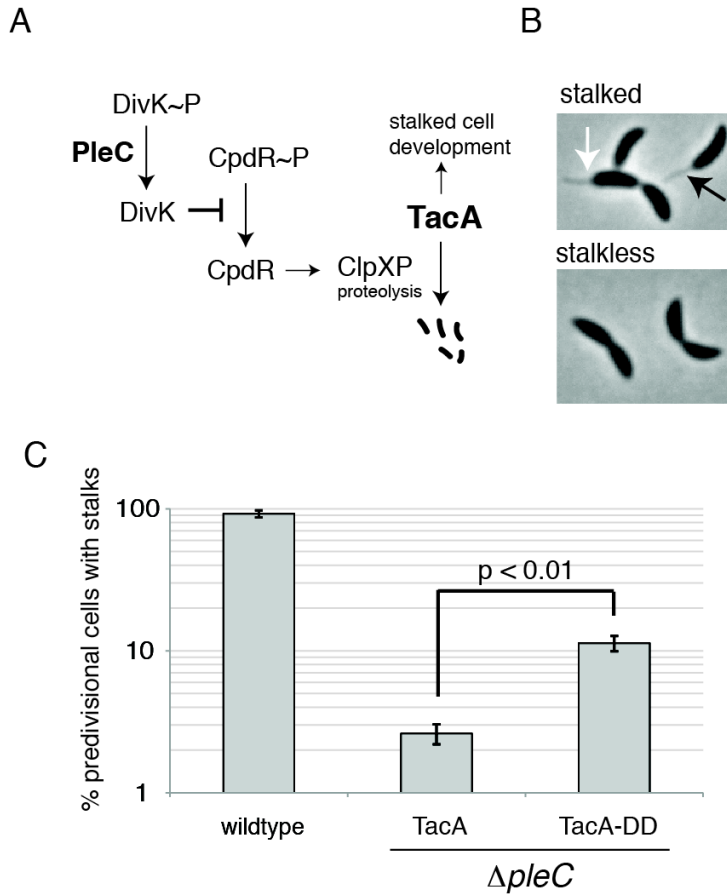
A. TacA is recognized by ClpXP *in vitro* and mutating C-terminal residues to Asp-Asp inhibits proteolysis. In these gels, overlapping bands corresponding to ClpX and creatine kinase are marked along with the purified TacA proteins. B. M2-FLAG epitope tagged TacA (M2-TacA) expressed from an inducible plasmid is degraded *in vivo* in a CpdR-dependent fashion following shift to a noninducing media. Representative western blot is shown here; quantification of replicates can be found in Figure S4\*. C. Western blots against the M2FLAG epitope and CtrA in synchronized population of wildtype cells shows that M2-TacA is degraded in a cell-cycle dependent fashion, while M2-TacADD is not degraded. Additional replicate illustrating M2-TacA degradation is shown in Figure S4\*. D. Loss of CtrA after antibiotic mediated shutoff of synthesis in cells expressing M2-TacADD compared to cells expressing M2-TacA. Upper panel shows a representative western blot, lower graph represents averages of biological replicates (n=4). Error bars are standard errors of the mean.

\* direct reference to Bhat et al., *Mol Microbiol.* 2013 Jun;88(6).

### 3.4 Discussion

Regulated proteolysis is essential for biological processes in all organisms and ClpP associated proteases contribute to a large fraction of protein degradation in bacteria [1]. In *C. crescentus*, control of proteolysis during cell cycle is critical for timing of development, DNA replication, and cell division [32]. By using a ClpP trapping approach, we now report a number of potential ClpP candidates that include known substrates (such as the master regulator CtrA), factors known to be ClpP substrates in other bacteria (such as LexA and FtsZ), and several novel ClpXP dependent proteolysis targets (such as TacA and IbpA). Some of these validated *Caulobacter* ClpXP substrates are targeted for degradation in other bacteria by other energy-dependent proteases (e.g., IbpA is degraded by Lon in *E. coli* [35]). Interestingly, CC0360, a putative ornithine decarboxylase, is degraded rapidly by full-length ClpX from *Caulobacter* and the eukaryotic ornithine decarboxylase is a rapidly degraded ubiquitin independent substrate of the 26S proteasome [36]. Biological networks rely on rapidly changing protein levels for effective signaling and the response in a protein's concentration given changes in its synthesis rate is more rapid when the half-life of that protein is short [37]. Thus, our work suggests that some proteins may be rapidly degraded regardless of the organism or proteolytic pathway involved because there is a universal need for their dynamic responses.

ClpXP mediated degradation of protein substrates often requires small peptide motifs that are directly recognized by the protease [1] and a classification of these motifs has been proposed [12]. We tested the conservation of these protease recognition motifs in our candidate pool and found that while motifs can direct degradation of some substrates, the simple presence of a motif is insufficient to predict direct recognition by ClpXP. We also found that, unlike *E. coli*, *Caulobacter* viability requires a function unique to the full-length ClpX protein. Although a variant of ClpX lacking its N-terminal zinc-binding domain can readily degrade substrates such as ssrA-tagged proteins and CtrA, this variant is incapable of supporting viability in *Caulobacter*



**Figure 3.5 Stabilization of TacA partially rescues stalk formation in  $\Delta pleC$  cells.**

A. Cartoon of PleC dependent TacA degradation. PleC dephosphorylates DivK. Dephosphorylated DivK inhibits CpdR dephosphorylation indirectly through CckA/ChpT (not illustrated here). Dephosphorylated CpdR promotes ClpXP degradation of TacA. Thus, loss of PleC would result in more dephosphorylated CpdR and faster TacA degradation. B. Representative images of wildtype cells (upper) with stalks marked with white arrows (predivisional cell) or black arrows (stalked cell) and stalkless  $\Delta pleC$  predivisional cells (lower). C. Stalk formation is partially recovered in  $\Delta pleC$  cells expressing stabilized TacA. Quantification of predivisional wildtype cells,  $\Delta pleC$  strains expressing TacA as the sole variant, or  $\Delta pleC$  strains expressing TacA-DD as the sole variant. Error bars are standard errors, p-value is calculated from a two-tailed Welch's t-test. Stalk elongation during phosphate limitation is also more pronounced in  $\Delta pleC$  cells expressing stabilized TacA (Figure S5\*).

\* direct reference to Bhat et al., Mol Microbiol. 2013 Jun;88(6).

and poorly degrades some of the validated ClpXP substrates presented in this work. Together, these biochemical observations demonstrate the utility and limitations of recognition motifs for substrate recognition and reveal a crucial role for the N-terminal domain of the *Caulobacter* ClpXP in substrate recognition and *in vivo* function.

The characteristic swarmer to stalked cell transition in *Caulobacter* requires a substantial reorganization of the proteome and we identified a number of cell stage specific factors in our trapping studies including the  $\sigma^{54}$ -dependent response regulator TacA, several chemotaxis proteins (CheR, CheW, CheD, and McpJ), and flagellar regulators (FlaF and FliM). Our results show that TacA is directly recognized by ClpXP *in vitro* but degradation *in vivo* requires CpdR. CpdR is needed for degradation of all known ClpXP substrates *in vivo* and dephosphorylation of CpdR increases degradation of these substrates [5,16,30]. Phosphorylation of CpdR occurs via the CckA/ChpT phosphotransfer pathway [30] and CckA is repressed by phosphorylated DivK [33]. The PleC phosphatase dephosphorylates DivK, and as a consequence, loss of *pleC* results in more dephosphorylated CpdR [33] and presumably would increase degradation of ClpXP substrates, such as TacA. Because  $\Delta pleC$  cells lack stalks [34] and as TacA is critical for stalk formation [7], it is tempting to speculate that prolific degradation of TacA is responsible for this absence of stalked cells. In support of this model, we found that stabilization of TacA partially restored stalked cell formation (Figure 3.5), although the lack of complete restoration implicates the role of additional factors as well.

Why does stabilization of TacA cause increased degradation of CtrA? TacA influences asymmetric cell division in *Caulobacter* in part through synthesis of SpmX which is needed for the proper accumulation and activation of the histidine kinase DivJ that phosphorylates DivK [29]. As mentioned above, DivK promotes CpdR and CtrA dephosphorylation by inhibiting the kinase activity of CckA [33], unleashing CpdR's ability to activate ClpXP [5,16,30] but blocking

CtrA's activity as a transcription factor [7,38]. One intriguing possibility is that TacA promotes CpdR dephosphorylation, perhaps through SpmX, effectively stimulating its own degradation and that of CtrA. In this type of negative feedback loop, increasing TacA through stabilization may result in prolific CpdR activation that could underlie the increased degradation of CtrA.

### **3.5 Acknowledgements**

We thank U. Jenal, J. Gober, and Y. Brun for providing reagents and strains; and L. Francis, M. Laub for comments. N.B. and P.C. were funded by NIH grant R00GM084157. P.S. was also supported by a HHMI Summer Research Fellowship for Undergraduates. We especially thank K. Green (University of Massachusetts, Worcester) for all of her advice and aid in mass spectrometry and protein identification.

### **3.6 Material and Methods**

#### **3.6.1 Plasmids and strain construction**

Standard techniques were used to manipulate *C. crescentus*. All *C. crescentus* strains were grown at 30°C in PYE with the appropriate antibiotics and sugars [39]. Briefly, we generated the proteolytic inactive ClpPtrap by mutating the active site serine to alanine (S98A) and appending a tandem affinity tag (his<sub>6</sub>-TEV-M2FLAG) to the C-terminus of ClpP. Low copy plasmids expressing ClpPtrap or wildtype affinity tagged ClpP were transformed into a strain whose sole, genomic copy of ClpP was driven by the xylose promoter [3] to generate trapping or control strains. ClpP substrate candidates were cloned with appropriate primers into either pET23SUMO plasmids for recombinant protein expression in *E. coli* [40] or into pENTR plasmids for Gateway-based cloning into expression plasmids for either *E. coli* or *C. crescentus* [39]. ΔNClpX (removing residues 1-58) and full length ClpX were expressed in ClpX depletion strains [3] using low copy plasmids downstream of the *clpX* promoter [41]. *Caulobacter* strains

harboring alleles of TacA-DD were generated by transforming wildtype or  $\Delta pdeA:tet$  [39] with pENTR TacA-DD. Because these promoter-less plasmids cannot replicate in *Caulobacter*, antibiotic resistance is gained only if they are integrated. Subsequent screening by PCR verified that plasmids were inserted at the correct locus downstream of the native *tacA* promoter.

### 3.6.2 Purification of ClpP trap and candidate identification

Detailed procedures for ClpPtrap expression and purification are given in the Supplemental Information. Briefly, sequential affinity columns were used to purify ClpPtrap after depletion of endogenous ClpP, preliminary validation of the trap was confirmed by western analysis against the known substrate CtrA. Trapped substrates were separated by 15% SDS-PAGE, gel slices (~1-5 mm) were excised, digested with trypsin and analyzed by mass spectrometry (Proteomics and Mass Spectrometry Facility, University of Massachusetts, Worcester).

### 3.6.3 Protein purification and degradation assays

Detailed information is given in Supplemental Information. Untagged *C. crescentus* ClpX and  $\Delta N$ ClpX were purified using a similar protocol as that described for *E. coli* ClpX [12]. Recombinant his-tagged *C. crescentus* ClpP was purified as described [27]. Candidates from the ClpP trap were expressed either as his-tagged constructs [39] or as his-SUMO-tagged constructs [40] and purified accordingly. Degradation reactions were performed in H-buffer (20 mM HEPES, 100 mM KCl, 10 mM MgCl<sub>2</sub>, 10% glycerol, pH 7.5; and an creatine kinase based ATP regeneration mix) at 30°C [27]. In a typical timecourse, aliquots at specific times were added to SDS-PAGE loading dye to quench the reaction and snap frozen on dry ice. Samples were heated at 95°C for 5 min or 65°C for 10 min and resolved by 12% or 15% SDS/PAGE gels. Coomassie stained gels were quantified by measuring intensity of substrate bands using ImageJ (NIH).



#### **3.6.4 *In vivo* stability and synchrony experiments**

For asynchronous *in vivo* degradation assays, cells with plasmids expressing epitope tagged TacA from xylose promoters were grown in PYE with xylose (0.1%) and spectinomycin (25µg/µl) overnight. In the morning the cultures were back diluted into the same media, grown until exponential growth, after which cells were harvested, washed twice in fresh PYE medium and resuspended in PYE-glucose to initiate the timecourse. Protein levels were assayed by Western blotting using antibodies against the M2-FLAG epitope (Sigma-Aldrich). For synchronized growth, *Caulobacter* were grown to an OD600 of 0.3-0.5 in PYE medium with the appropriate antibiotic, swarmer cells were isolated by Percoll density centrifugation, and cells were released into the same media. For CtrA stability experiments, swarmer cells were isolated and resuspended in pre-warmed PYE media containing 30µg/ml chloramphenicol to shut off synthesis.

CC / CCNA	gene	reference	CC / CCNA	gene	reference
CC_0008	dnaA	2, 6	CC_2540	ftsZ	1, 2, 5, 6
CC_0011	dnaJ	2, 5, 6	CC_2541	ftsA	2, 5
CC_0042	infB		CC_2546	murC	6
CC_0160	gyrB	2, 5	CC_2700		2
CC_0267	dnaX		CC_3035	ctrA	1, 2, 4, 6
CC_0360			CC_3145	mcpJ	2
CC_0435	cheR	1, 2	CC_3192		
CC_0695	mutL		CC_3315	tacA	2
CC_0808			CC_3448	atpG	
CC_1005			CC_3492	nrdA	6
CC_1087	recA	2, 6	CC_3494		
CC_1118			CC_3527	sdhA	3
CC_1961	clpX	5	CC_3691		2
CC_2102	sspB	2	CCNA_00466	rfaG	
CC_2258	ibpA	2, 4	CCNA_00469		
CC_2468	clpA	2	CCNA_00999		3, 4

**Table 3.1 Subset of trapped ClpP candidates.**

Subset (32/127) trapped candidates that satisfy high stringency requirements of >5 unique peptides in replicate trapping experiments, removing proteins that copurified with active ClpP, and eliminating proteins that are overly abundant in whole cell extracts (see Supplemental Information). Candidates are listed by CC numbers from CB15 (GenBank ID AE005673) or CCNA numbers from NA1000 (GenBank ID CP001340). Where known, gene names are also listed. References are: (1) proteins known to be unstable during cell cycle progression [23]. (2) genes known to be transcriptionally regulated during cell cycle [42]. (3) proteins known to be tagged by the trans-translation pathway [18]. (4) proteins that end in either AA or SA at their extreme C-terminus. (5) putative ClpP substrates identified in other bacteria [12,19,21]. (6) proteins whose levels decrease during carbon starvation [22]. An expanded version of this table with all ClpP candidates (127) can be found in Supplemental Information (Table S1\*).

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\* direct reference to Bhat et al., *Mol Microbiol.* 2013 Jun;88(6).

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#### **4. CRITICAL CLAMP LOADER PROCESSING BY AN ESSENTIAL AAA+ PROTEASE IN *CAULOBACTER CRESCENTUS***

*as written by Vass R.H. and Chien P., PNAS 2013*

##### **General Chapter Overview**

After the initial proteomic screen for identifying and characterizing novel ClpA/X(P) targeted substrates, one particular protein stood out from the several other substrates that I was characterizing at the time. Usually during gel-based, *in vitro* degradation assays, substrates that are recognized by the unfoldase pair of ClpAP/XP are destroyed. Starting levels of protein subjected to ClpP proteolysis are completely degraded given enough energy currency (non-limiting amounts of ATP) and time. Finding DnaX to be enriched by the ClpP was surprising as DnaX was not previously known to be targeted for ClpP destruction (as outlined in Chapter 2). Contrary to proteins degraded by ClpXP, DnaX was not fully degraded. Exposure of the DnaX substrate to the protease consistently left two distinct smaller size fragments that remained stable over time, suggesting certain features of the protein inhibited full unraveling and degradation by the normally processive ClpXP system. This chapter highlights both the biochemical and physiological characterization of what causes incomplete destruction and what relevance this incomplete proteolysis of DnaX has inside *Caulobacter* cells.

My major contributions to this chapter centers on performing all of the biochemistry and molecular biology experiments and generating figures required for this work.

## 4.1 Abstract

Chromosome replication relies on sliding clamps that are loaded by energy dependent complexes. In *E. coli*, the ATP-binding clamp loader subunit DnaX exists as both long ( $\tau$ ) and short ( $\gamma$ ) forms generated through programmed translational frameshifting, but the need for both forms is unclear. Here, we show that in *Caulobacter crescentus*, DnaX isoforms are unexpectedly generated through partial proteolysis by the AAA+ protease ClpXP. We find that the normally processive ClpXP protease partially degrades DnaX to produce stable fragments upon encountering a glycine-rich region adjacent to a structured domain. Increasing the sequence complexity of this region prevents partial proteolysis and generates a  $\tau$ -only form of DnaX *in vivo* that is unable to support viability on its own. Growth is restored when  $\gamma$  is provided in trans but these strains are more sensitive to DNA damage compared to strains that can generate  $\gamma$  through proteolysis. Our work reveals a novel mode of partial processing by the ClpXP protease to generate DnaX isoforms, demonstrates that both  $\tau$  and  $\gamma$  forms of DnaX are required for *Caulobacter* viability, and identifies a role for clamp loader diversity in responding to DNA damage. The conservation of distinct DnaX isoforms throughout bacteria despite fundamentally different mechanisms for producing them suggests there may be a conserved need for alternate clamp loader complexes during DNA damaging conditions.



## 4.2 Significance Statement

Chromosome replication requires sliding clamps that are loaded by clamp loader complexes. In *E. coli*, these complexes exist as long ( $\tau$ ) and short ( $\gamma$ ) forms produced by programmed ribosome frameshifting during translation of the DnaX clamp loader subunit. Because many bacterial *dnaX* genes lack a frameshift site and because *E. coli* does not require  $\gamma$  for normal growth, the biological significance of both forms has been unclear. Here we show that in *Caulobacter crescentus*, partial proteolysis of DnaX by the ClpXP protease generates shorter  $\gamma$ -like forms that are required for normal growth and DNA damage tolerance. This novel route of  $\gamma$ -production illustrates that clamp loader diversity is common in bacteria and, given our results, may generally impact replication and repair.

### 4.3 Introduction

All cellular life uses energy dependent proteolysis to destroy undesired proteins [1]. In bacteria, the ATP-dependent oligomeric protease ClpXP recognizes substrates critical for cell division [2], response to DNA damage [3], and transition to stationary phase [4-6]. The ClpX unfoldase recognizes peptide motifs within targets, then hydrolyzes ATP to unfold and translocate these substrates into the inner chamber of the ClpP peptidase for proteolysis [1]. Although ClpXP can completely degrade proteins that differ widely in stability and length [1,7], incomplete proteolysis has been reported *in vitro* with engineered substrates particularly when ATP hydrolysis becomes limiting [8] or when substrate load is excessive [9-11]. However, it is unknown whether such partial proteolysis naturally occurs for ClpXP substrates *in vivo* or has any biological consequence in bacteria.

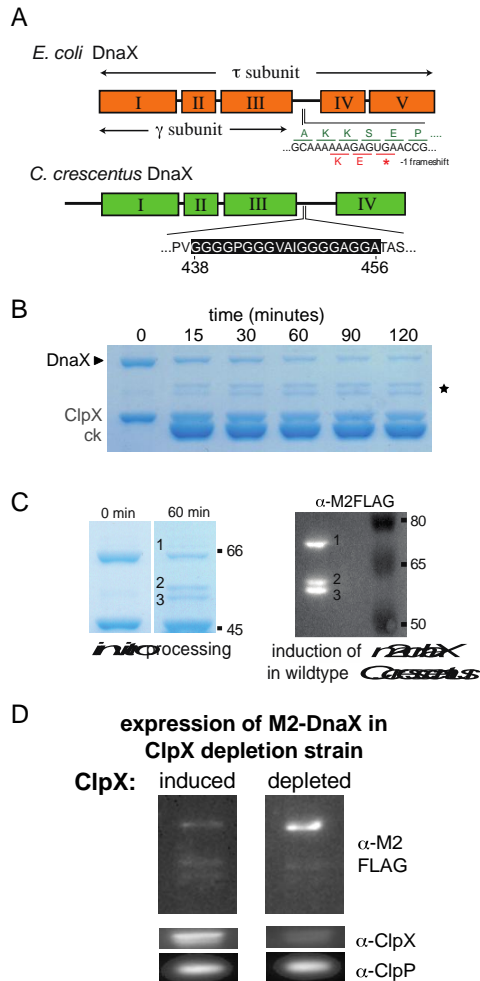
Processive replication of chromosomes requires energy dependent loading of ring-shaped sliding clamps that tether replicative polymerases to their templates. The pentameric bacterial clamp loader complex contains one copy each of the HolA and HolB subunit, and three copies of the ATP-hydrolyzing DnaX subunit [12] that exists as two distinct forms in *E. coli*, a full-length  $\tau$ -form and a shorter  $\gamma$ -form generated by programmed ribosomal frameshifting [13-15] (Figure 4.1A). Gamma ( $\gamma$ ) contains amino-terminal domains needed for binding ATP, the sliding clamp, and other subunits, reviewed in [12]. Clamp loader complexes with only  $\gamma$  forms of the DnaX subunits are able to assemble clamps onto appropriate templates *in vitro* [12]. However, the longer  $\tau$  form contains additional C-terminal domains essential for binding replisome components such as the DnaB helicase and the  $\alpha$ -subunit of Pol III during chromosomal replication *in vivo* [16-19]. The need for both forms has been debated since their discovery and it is unclear how, or even if,  $\gamma$  is generated in bacteria such as *C. crescentus*, where the *dnaX* ortholog lacks an obvious ribosomal frameshifting site (Figure 4.1A).

Here, we show that in *C. crescentus*, DnaX is processed by the essential protease ClpXP to generate a shorter,  $\gamma$ -like form both *in vivo* and *in vitro*. Generating the shorter form requires a stably folded domain, a 'slippery' glycine rich tract, and a protease recognition site that together result in the premature release of partially processed DnaX. We find that eliminating this partial processing via mutation of the slippery tract produces a  $\tau$ -only form of DnaX that is incapable of supporting normal growth, but can sustain viability when  $\gamma$  is expressed in trans. Finally, we show that processing of DnaX is critical for responding properly to DNA damaging conditions. Taken together, these results suggest that DnaX subunit diversity is a critical feature of the clamp loader complex and demonstrate a novel route to generate such diversity.

## 4.4 Results

### 4.4.1 DnaX is partially processed by ClpXP

We recently identified DnaX as a putative ClpXP substrate through an unbiased proteomic trapping approach [20]. ClpXP is a highly processive enzyme that normally completely degrades its target substrates [1,7]. In contrast, ClpXP proteolysis of DnaX generates stable truncation products (Figure 4.1B). Mass spectrometry revealed that these products contained intact N-termini and estimated molecular weight by SDS-PAGE corresponds to fragments composed of the first two-thirds of DnaX (Figure 4.1C), similar in size and domain structure to the *E. coli*  $\gamma$  [13-15]. Consistent with this interpretation,



**Figure 4.1 DnaX is partially processed by ClpXP; substrates are recognized by ClpX, unfolded and translocated in an ATP dependent process to the peptidase ClpP, which degrades the target.**

A. DnaX from *E. coli* and *C. crescentus* share similar N-terminal domains, but are less conserved at the C-terminus. The *E. coli* ortholog contains a hexa-adenine stretch that promotes ribosomal frameshifting during translation to generate the  $\gamma$  form. The *C. crescentus* ortholog lacks an obvious frameshifting sequence, but contains a glycine-rich region following the conserved N-terminal domains. B. Purified DnaX processing by the ClpXP protease *in vitro* (ck: creatine kinase). Truncation products are marked with an asterisk. C. Fragments generated by incubating purified DnaX with ClpXP for 60 minutes (left) or multiple forms of M2-tagged DnaX expressed in wildtype *C. crescentus* as detected by anti-FLAG (right). Numbers on right correspond to molecular weight markers. D. Decreasing ClpX levels directly impacts DnaX processing. Levels of ClpX and ClpP after 3 hours of growth in either inducing (left) or depletion (right) conditions in a ClpX depletion strain (21). In this case M2-DnaX was induced at 2 hours and detected by anti-FLAG.

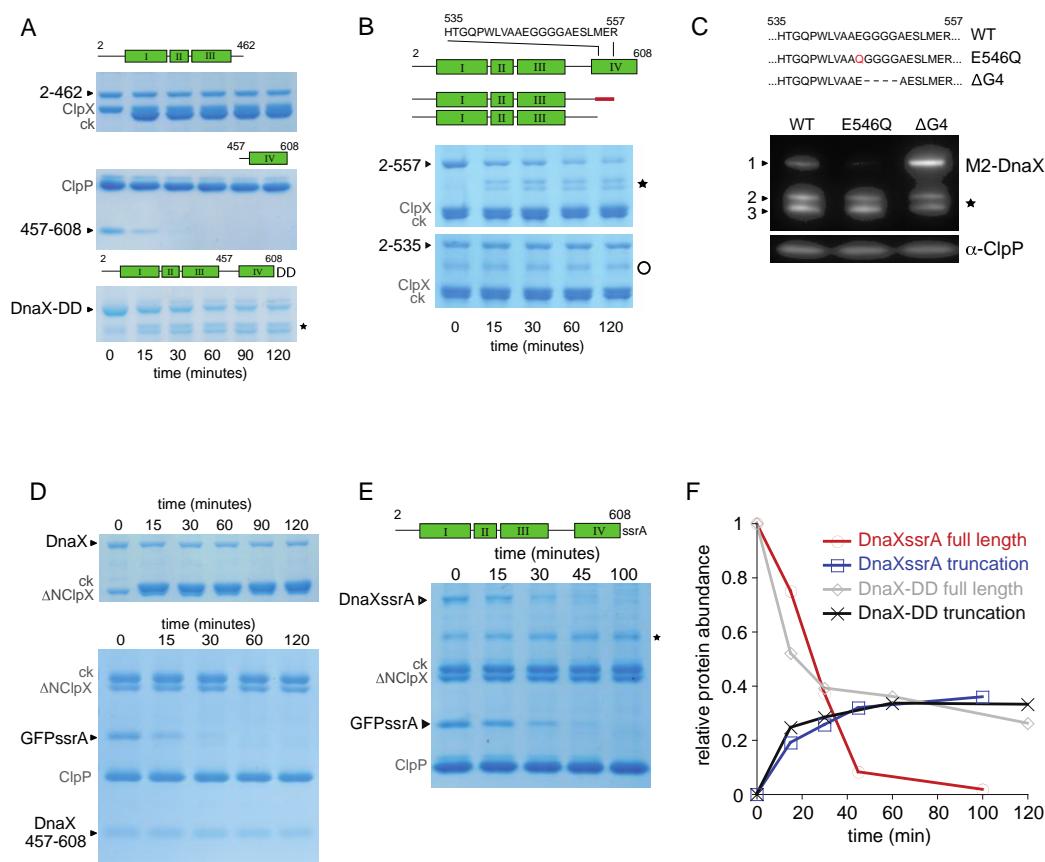
induction of an N-terminally M2-FLAG tagged DnaX (*m2-dnaX*) fusion produces DnaX fragments containing the N-terminal epitope with similar size distribution as those derived from ClpXP proteolysis *in vitro* (Figure 4.1C). The amount of processing was similar *in vitro* and *in vivo*, with 35-38% of full-length protein remaining in both cases. To determine if proteolysis was important for DnaX fragment formation *in vivo*, we expressed *m2-dnaX* in a strain where the sole chromosomal copy of *clpX* is under inducible control [21]. In this strain, ClpX is present only under inducing condition and is rapidly lost in when the inducer is withdrawn (Figure S4.1). When *clpX* is induced, fragments of M2-DnaX are produced similar as those seen in wildtype cells (Figure 4.1C,D). However, upon ClpX depletion, M2-DnaX principally exists as the full-length form (Figure 4.1D), supporting our biochemical results that ClpXP generates these fragments *in vitro*. Based on these results, we next addressed what elements of DnaX were needed for protease recognition.

#### **4.4.2 DnaX processing by ClpXP requires internal residues**

ClpXP often recognizes proteins via recognition motifs at the extreme N- or C-terminus of a target [1], therefore we initially searched for similar protease recognition sites in DnaX. While the N-terminal portion of DnaX was stable, we found that the isolated folded C-terminal domain IV of DnaX was rapidly degraded suggesting that ClpXP recognizes the C-terminus of DnaX (Figure 4.2A; Figure S4.2). However, addition of aspartate substitution mutations that are known to block ClpXP binding of carboxy-terminal motifs in many substrates [20,22] failed to eliminate DnaX processing (Figure 4.2A). Instead, truncations of DnaX suggested that residues 535-557 were important for recognition of DnaX by ClpXP (Figure 4.2B). Internal recognition motifs for ClpXP substrates have been previously reported [23] and in agreement with the important role of this internal region, mutations or deletions within residues 535-557 of DnaX alter the processing of the full length DnaX *in vivo* (Figure 4.2C and Figure S4.3). Taken together with the truncation results, these data are consistent with a model where ClpXP initiates

processing from this internal region during recognition of the full-length protein. However, it is also possible that this internal region acts in trans to facilitate recognition of some other portion of DnaX by ClpXP, such as seen in the case of MuA [24] and UmuDD' [25] where elements distinct from the degradation initiation site contribute to proteolysis. Next we determined which portion of ClpX is needed to interact with DnaX.

AAA+ proteases contain highly similar ATPase domains that provide chemical energy for the unfoldase activity and family specific domains needed for particular functions [1]. The ClpX family of unfoldases is distinguished by an N-terminal domain that is required for adaptor binding and recognition of some substrates, but dispensable for degradation of other targets, such as ssrA-tagged proteins [26]. Interestingly,  $\Delta$ NClpXP could not process the full-length DnaX or recognize the C-terminal domain IV even in conditions where another substrate (GFPssrA) was degraded (Figure 4.2D). Thus, the need for the internal recognition motif of wildtype DnaX for degradation also requires the characteristic domain of ClpX family members. To address if this family specific interaction was required for partial processing, we forced  $\Delta$ NClpXP to recognize DnaX by appending an ssrA tag to the C-terminus of DnaX. This construct was readily degraded by  $\Delta$ NClpXP and, importantly, accumulated intermediate fragments to a similar



**Figure 4.2 DnaX contains an internal ClpXP recognition motif.**

A. Domains of DnaX or full-length DnaX containing carboxy-terminal di-aspartates incubated with ClpXP *in vitro*. Accumulating truncation products of mutant full-length DnaX are marked by an asterisk. B. Serial C-terminal truncations revealed an internal ClpXP recognition site in domain IV, which resides between residues 535-557. Accumulating truncation products are marked with an asterisk, n.b. a truncation-sized contaminant (circle) is present in the lower gel but does not accumulate over time. C. Alterations made on residues contained within this region change both the distribution and stability of full-length protein seen *in vivo*. Disrupting amino acid residues within region 535-557 directly affects the stability of DnaX by changing both stability and frequency of processing (see Figure S4.3). D. Neither full-length DnaX nor the C-terminal domain of DnaX is recognized by ΔNClpXP, although this protease is fully active for degradation of an ssrA tagged substrate (GFPssrA). E. DnaX appended with an ssrA tag is recognized by ΔNClpXP, asterisk marks buildup of intermediate. F. Quantification of full-length or truncation products over the time course of degradation. Levels were normalized to the amount of initial full-length protein.

degree as that seen with the wildtype protease/substrate (Figure 4.2E,F). Therefore, the partial processing of DnaX is independent of the precise nature of the recognition site and must rely on other features.

#### **4.4.3 Partial processing of DnaX requires folded domains adjacent to a glycine-rich sequence**

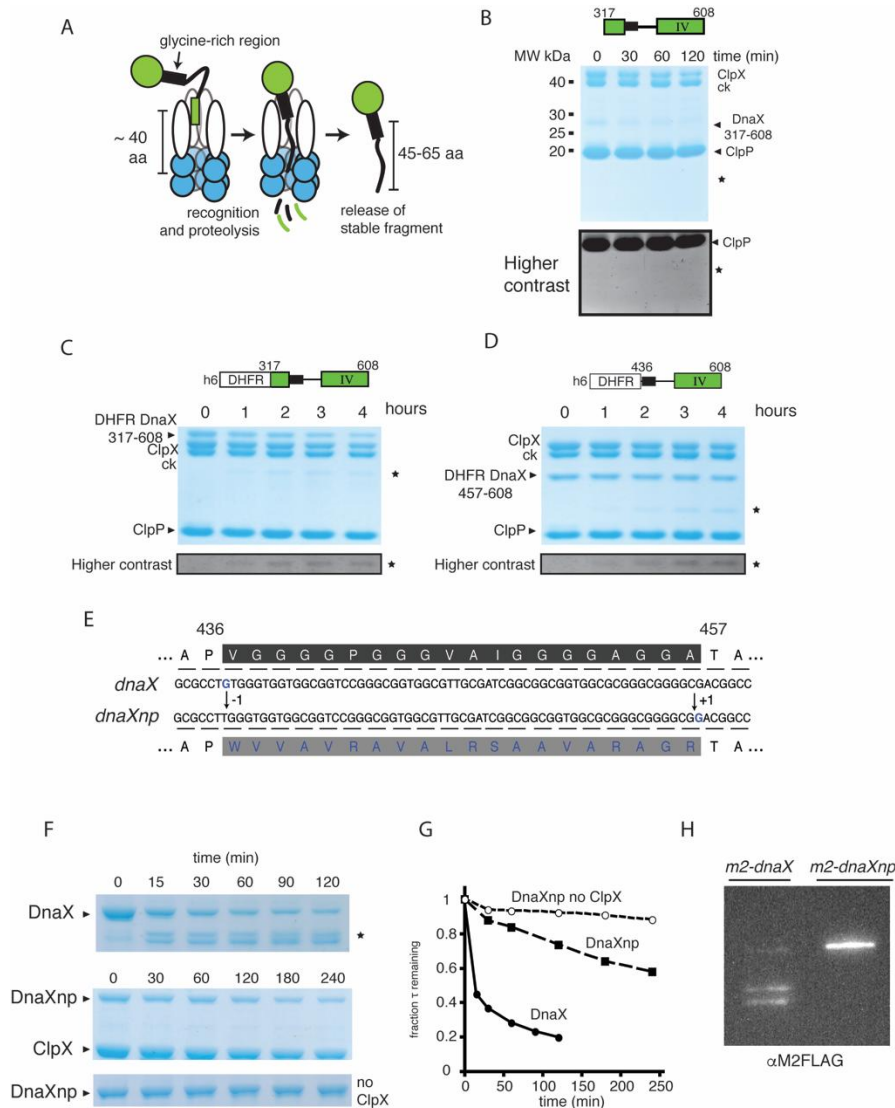
The *C. crescentus* *dnaX* encodes a glycine rich sequence centered at residue 445 in an otherwise poorly conserved region among  $\alpha$ -proteobacterial orthologs (Figure 4.1A). In our *in vivo* studies, all the observed DnaX fragments were detected by an N-terminal epitope tag (Figure 4.1C) suggesting that these fragments resulted from proteolysis of the C-terminal domains. In support of this model, peptides derived from trypsinization/mass spectrometry of the *in vitro* generated DnaX fragments were consistent with retention of the N-terminus but loss of C-terminal residues (Supplemental Figure S4.3C) and Edman degradation sequencing of the smallest truncated product confirmed the presence of the native N-terminus (ADHDD). Based on these results and the estimated molecular weight from SDS-PAGE (Figure 4.1C; Supplemental Figure S4.3C) DnaX truncations generated *in vitro* are consistent with fragments that contain the intact N-terminus and terminate between residues 490 and 520.

Prior studies have suggested that the distance between the ClpX unfoldase pore and active site of the ClpP peptidase spans ~40 residues of a fully extended polypeptide [10,27] consistent with the observed tails of DnaX fragments if ClpX stalls at the glycine-rich region (Figure 4.3A). Interestingly, the 26S proteasome is known to stall at glycine-rich regions during ubiquitin-mediated proteolysis if those regions are adjacent to tightly folded domains [28,29] and more recently, a similar effect has been shown with engineered substrates of ClpXP *in vitro* [30]. We addressed if processing of DnaX followed similar rules by generating a construct containing residues 317-608 that removes the oligomerizing N-terminal domains of DnaX [31] but preserves the glycine-rich region and residues 535-557 that are critical for ClpXP degradation. ClpXP



could degrade this construct, but truncation products did not accumulate (Figure 4.3B). If the structural stability adjacent to the glycine-rich region is important for stalling, then addition of a folded domain should restore stalling. Indeed, when we appended the well-folded *E. coli* DHFR protein (which is poorly degraded *in vitro* [27]) to fragments containing the glycine-rich region, ClpXP processing once again produces stable truncation products (Figure 4.3C,D).

We next specifically addressed the role of the glycine-rich region by recoding this segment to a more complex composition in the hope of generating a "non-processed" protein (*dnaXnp*; Figure 4.3E). By increasing the variety of amino acids at this region without dramatically changing mRNA content, this approach also allows us to isolate effects due to polypeptide composition from RNA-specific effects (such as ribosomal frameshifting). Purified DnaXnp was degraded slowly *in vitro* but, importantly, did not accumulate significant amounts of truncated species even after full-length loss had proceeded to the same extent as the wildtype (Figure 4.3F,G; compare DnaX at 15 min, with DnaXnp at 240 min). Expression of the *m2-dnaXnp* variant showed that the full-length form is exclusively made *in vivo* (Figure 4.3H). Taken together with the previous results, we conclude that the natural partial processing of DnaX requires both a tightly folded domain and a glycine-rich region, similar to those requirements for the 26S proteasome [28,29]. We note that because DnaXnp is degraded slowly, it is possible that



**Figure 4.3 Partial processing of DnaX requires folded domains adjacent to a glycine-rich sequence.**

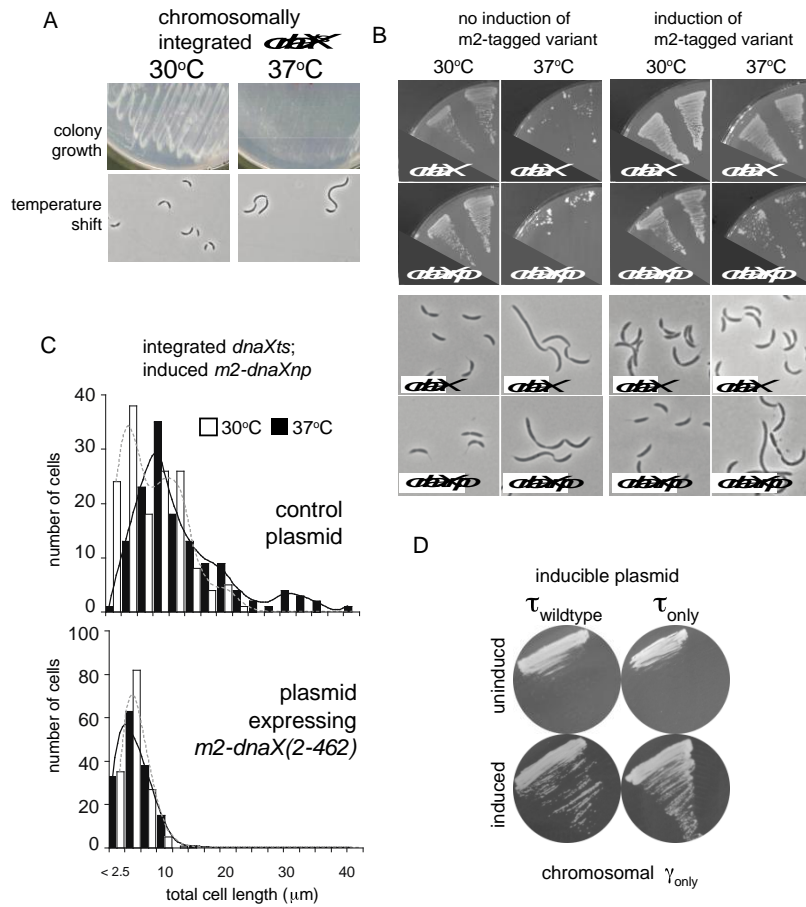
**A.** Model for partial processing of DnaX (green) by ClpXP, showing glycine-rich region and stable fragment size as determined by mass spectrometry. **B.** Domain of DnaX containing ClpXP recognition sequence, but lacking the N-terminal regions, is degraded, but does not build up truncation products. Predicted truncation size is shown by asterisk. **C,D.** Appending the stably folded *E. coli* DHFR domain to the N-terminus of DnaX fragments with the glycine-rich region and the ClpXP recognition site results in buildup of truncations (marked with asterisk). In **B-D**, higher contrast images for the marked regions are shown below each gel. **E.** Illustration of glycine-rich region and the -1/+1 mutations to generate *dnaXnp* (encoding nonprocessed DnaX). **F.** DnaXnp and DnaX processing by ClpXP *in vitro*, asterisk marks buildup, quantified in **G**. Note the lack of truncation accumulation in DnaXnp. **H.** Expression of *m2-dnaXnp* and *m2-dnaX* from low copy plasmids in wildtype *Caulobacter*.

partial fragments of DnaXnp are transiently generated but are degraded so rapidly relative to full-length protein that they are undetectable by our methods.

#### 4.4.4 DnaX diversity is critical for normal bacterial growth

Although the existence of the  $\tau$  and  $\gamma$  forms was originally described over twenty years ago [13-15] the biological relevance of the shorter form remains unclear. Eliminating the programmed frameshift site of *dnaX* in *E. coli* produced a presumably  $\tau$ -only form *in vivo* that was capable of supporting normal growth [19]. Similarly, the lack of programmed translational frameshift sites in several *dnaX* orthologs raises the question of whether  $\gamma$  even exists in these bacteria although in one such case,  $\gamma$  appears to be generated through transcriptional slippage [32]. Our identification of a novel pathway for  $\gamma$  formation via ClpXP proteolysis led us to reinvestigate the importance of different DnaX forms *in vivo*.

We produced a conditional allele of *dnaX* by mapping known temperature sensitive *E. coli* *dnaX* mutations [19] to equivalent positions in *C. crescentus* *dnaX*. When present as the sole copy, one of these variants (*dnaX<sup>ts</sup>*; A175D) sustained growth at 30°C, but not at 37°C, with significant morphological defects seen after shifting cells growing at 30°C to 37°C (Figure 4.4A). Expression of *m2-dnaX* from a plasmid in this background restored robust growth and normal morphology at restrictive temperatures (Figure 4.4B). In contrast, expression of *m2-dnaXnp* could not restore normal morphology after a shift to 37°C and produced colonies at higher temperatures only after prolonged incubation (Figure 4.4B). Subsequent tests showed that most of these colonies had lost the temperature sensitive phenotype and likely arose through recombination of the genomic *dnaX<sup>ts</sup>* with the plasmid in these *rec+* strains (Figure S4.4). As



**Figure 4.4 DnaX diversity is critical for normal bacterial growth.**

A. Strains with a temperature sensitive allele of *dnaX* (*dnaX<sup>ts</sup>*) grow poorly at 37°C (upper) and become filamentous after shifting to higher temperatures (lower), but grow normally at 30°C. B. (upper) *dnaX<sup>ts</sup>* strains with plasmids containing inducible epitope tagged DnaX variants, *m2-dnaX* or *m2-dnaXnp* (appropriate variant listed in each picture), grown at 30°C or 37°C in either inducing or noninducing conditions. (lower) Images of the same cells grown at 30°C, shifted to 37°C or maintained at 30°C for an additional 5 hours of growth. C. *dnaX<sup>ts</sup>* strains inducing *m2-dnaXnp* with an additional plasmid expressing the N-terminal,  $\gamma$ -form of DnaX (*m2-dnaX(1-462)*) or control plasmid. For each condition, total lengths of individual cells (n=150) were measured after shifting from 30°C to the indicated temperatures (see Methods). Envelopes of the distributions are outlined to aid in comparison. D. Growth of strains with  $\gamma_{\text{only}}$  *dnaX(1-462)* integrated at the chromosomal *dnaX* locus and DnaX variants expressed from a plasmid in either noninducing (upper) or inducing (lower) conditions.

predicted [19], neither expression of  $\gamma$  (*dnaX(1-462)*) nor the C-terminal  $\tau$ -specific region alone could complement *dnaX<sup>ts</sup>* in nonpermissive conditions (Figure S4.5). Unexpectedly, however, our results show that the  $\tau$ -form alone of DnaX is insufficient for supporting normal growth. The inability of *dnaXnp* to support normal growth could be explained by (i) the inability to process  $\tau$  to  $\gamma$  or (ii) the lack of  $\gamma$  itself. To distinguish between these possibilities, we tested if generating  $\tau$  and  $\gamma$  individually could complement the growth defects. We used the above described *dnaX<sup>ts</sup>* strain expressing *m2-dnaXnp* ( $\tau_{\text{only}}$ ) from a plasmid and transformed it with either an additional plasmid expressing *m2-dnaX(1-462)* ( $\gamma_{\text{only}}$ ) or a control plasmid. *dnaX<sup>ts</sup>* cells expressing  $\tau_{\text{only}}$  are slightly filamentous at 30°C and dramatically longer at 37°C (Figure 4.4C). However, expression of  $\gamma_{\text{only}}$  restores normal wildtype cell length at both restrictive and permissive temperatures (Figure 4.4C). As a further confirmation of these results, we replaced the chromosomal *dnaX* allele with  $\gamma_{\text{only}}$  in cells expressing either *m2-dnaX* or *m2-dnaXnp* from inducible plasmids to generate  $\tau_{\text{wildtype}}/\gamma_{\text{only}}$  and  $\tau_{\text{only}}/\gamma_{\text{only}}$  strains respectively. In both cases, the resulting strains grow normally with induction even though M2-DnaXnp remains full length in this background (Figure 4.4D; Figure S4.6). Taken together with the above results we conclude that both  $\tau$  and  $\gamma$  forms of DnaX are required for normal growth.

#### 4.4.5 Processing of DnaX is needed for a proper response to DNA damaging conditions

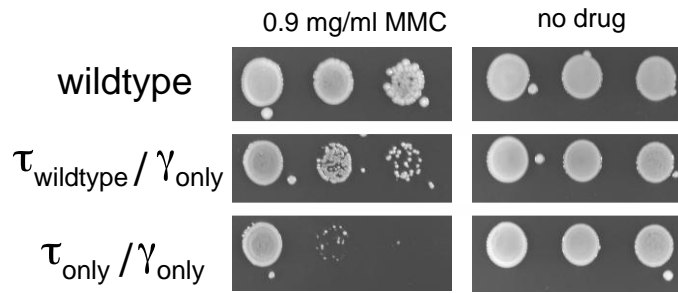
The biological role of the  $\gamma$ -form of the clamp loader is not known, but one speculation is that the  $\gamma$ -containing clamp loader complex may be beneficial during replication stress or DNA damaging conditions. For example, the tight binding of the  $\tau$ -specific elements of DnaX to the Pol III replicase components [17,18] could potentially inhibit the recruitment of alternative polymerases, such as those upregulated in response to DNA damaging conditions, to the

replication fork upon encountering template damage [16,33,34]. In *Caulobacter*, relieving this inhibition by removing the  $\tau$  specific portions through processing would aid in polymerase exchange during damage. We tested this by treating  $\tau_{\text{wildtype}}/\gamma_{\text{only}}$  and  $\tau_{\text{only}}/\gamma_{\text{only}}$  strains with mitomycin C (MMC), a DNA damaging agent. We found that the ability to process DnaX to the  $\gamma$  form is important for damage response as  $\tau_{\text{only}}/\gamma_{\text{only}}$  strains are more sensitive to MMC (Figure 4.5A). Alternative polymerases, such as those upregulated upon DNA damage, are often mutagenic due to their low fidelity and production of rifampicin resistant mutants upon damage has been used in *Caulobacter* to detect the activity of these polymerases [35]. In support of a model where DnaX processing is needed for the proper recruitment of these alternative polymerases,  $\tau_{\text{only}}/\gamma_{\text{only}}$  strains produce fewer UV-induced rifampicin resistant mutants than  $\tau_{\text{wildtype}}/\gamma_{\text{only}}$  strains (Figure 4.5B). Thus, although the constitutive presence of  $\tau$  and  $\gamma$  are sufficient for normal growth, the processing of DnaX appears needed for robust DNA damage responses.

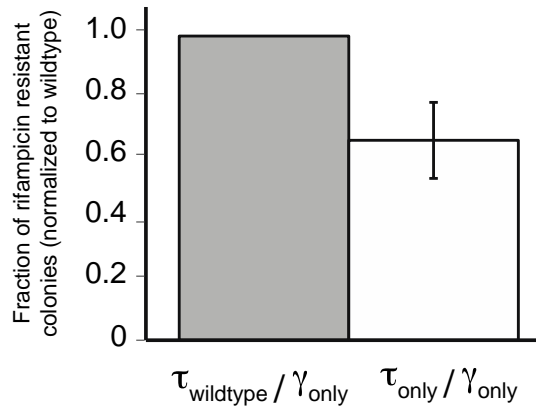
## 4.5 Discussion

Protein degradation by energy dependent AAA+ proteases is critical for maintaining and sculpting the proteome [1]. ClpXP is normally a highly processive protease that employs repeated cycles of ATP consumption to degrade proteins of varying sizes and stability. While ClpXP can fail on artificial substrates *in vitro*

A



B



**Figure 4.5 Dynamic production of  $\gamma$  is needed for response to DNA damaging conditions.**

A. Strains expressing  $\gamma_{\text{only}}$  from the chromosome and either  $\tau_{\text{wildtype}}$  or  $\tau_{\text{only}}$  from inducible plasmids are spotted on inducing plates containing mitomycin C (MMC) or no drug. Wildtype strains are shown for comparison. Cultures were serially diluted 10-fold from left to right in each panel. B. Individual cultures (n=10) of strains as described above were UV irradiated (10,000 mJ/cm<sup>2</sup>) recovered for 14 hours to fix mutations, then plated on rifampicin containing media and on nonselective media. Three separate experiments were performed and the fraction of rifampicin resistant colonies relative to wildtype was determined for each trial. The bar represents the average of these trials with standard error.

[9,11,30], our work represents the first observation of ClpXP naturally partially processing an endogenous substrate *in vivo*. We find that the requirements of (i) a protease recognition site, (ii) an intervening region of low complexity and (iii) a stably folded domain, mirror those elements required for stalling *in vitro* [29,30], supporting a model where constraints for partial proteolysis are conserved between prokaryotic and eukaryotic energy-dependent proteases. It therefore seems likely that partial processive proteolysis, such as that seen here for DnaX in *Caulobacter*, may be a general strategy in bacteria to produce increased functional diversity from a single gene product.

We were surprised to find that the shorter  $\gamma$  form of DnaX is required for normal growth in *C. crescentus*, in contrast to that reported in *E. coli* [19]. This suggests that an obligate  $\tau$ -only complex as proposed in *E. coli* [36] may not be a universal feature of bacterial replisomes. Furthermore, although strains expressing  $\tau$ -only variants of DnaX are viable in the presence of additional  $\gamma$ , these strains are unable to properly respond to DNA damaging agents and exhibit a reduced ability to use alternative error-prone polymerases upon UV radiation. Based on these results, the processing of DnaX seems critical for proper DNA damage response in *Caulobacter*. One speculative explanation is that the  $\tau$  form of DnaX binds so tightly to Pol III replicase components that it limits polymerase exchange during DNA damaging conditions. Processing of DnaX and removal of the  $\tau$ -specific portions would therefore release these components and promote an increased exchange of replisome components that may improve the response to DNA damage. However, it is important to note that in addition to its role during DNA damaging conditions, the  $\gamma$ -form of the DnaX must have a separate critical function during normal chromosome replication in *Caulobacter*, given that alternative polymerases are dispensable [35] during normal growth conditions.



Taken together, our work shows how a normally highly processive protease can partially degrade a substrate according to simple rules. In *Caulobacter*, this partial processing is used to produce multiple forms of the DnaX subunit of the clamp loader. The presence of multiple DnaX forms among many bacteria despite widely different production methods suggests a conserved need for both  $\tau$  and  $\gamma$  forms of the clamp loader, possibly in response to DNA damage as shown here.

#### **4.6 Acknowledgements**

The authors wish to thank all members of the Chien lab, L. Francis, E. Strieter, and B. Kelch for comments on the manuscript. Supplementary data reported in this paper can be found in the Supporting Online Material. P.C. and R.H.V. were funded by NIH grant R00 GM084157 and funds from the University of Massachusetts, Amherst. P.C. and R.H.V. designed experiments, R.H.V. performed experiments. R.H.V. and P.C. analyzed results and wrote the manuscript.

#### **4.7 Materials and Methods**

##### **4.7.1 *In vitro* degradation assays.**

SUMO-tagged DnaX variants, ClpP, and ClpX were purified following procedures as before [37]. Standard degradation reactions were performed in ClpXP degradation buffer (20 mM HEPES, 100 mM KCl, 10 mM MgCl<sub>2</sub>, 10% glycerol, pH 7.5; and an creatine kinase based ATP regeneration mix) and analyzed as before [37].

#### 4.7.2 Identification of processed DnaX fragments.

Fragments generated through *in vitro* degradation were subjected to trypsinization/mass spectrometry (University of Massachusetts, Worcester Medical School Proteomics and Mass Spectrometry Facility) and Edman degradation/sequencing (Tufts University Core Facility).

#### 4.7.3 Recombinant protein expression plasmids.

Primer sequences are designated as OPC followed by a unique number and can be found in Supplemental Information. Direct amplification of *Caulobacter crescentus dnaX* was from CB15N genomic DNA (*dnaX*, OPC 280/281). These primers incorporated an AgeI restriction site upstream of gene removing the start Met and placed a stop codon followed by an XhoI restriction site downstream. Insert was restriction digested and ligated into digested pET23-his<sub>6</sub>-SUMO vector via AgeI/XhoI sites. pET23-his<sub>6</sub>-SUMO appends an N-terminal his<sub>6</sub>-SUMO upstream of ligated gene for both initial NiNTA purification and final native protein state after removal of his<sub>6</sub>-SUMO tag. Amplification of individual inserts *dnaX*(317-608) (OPC 309/281), *dnaX*-DD (OPC 280/310), *dnaX*(457-608) (OPC 350/281), *dnaX*(1-462) (OPC 280/361), *dnaX*(2-535) (OPC 280/529), *dnaX*(2-556) (OPC 280/530), and *dnaX*-436-608 (OPC 592/281) were cloned from pET23-his<sub>6</sub>-SUMO-*dnaX* and ligated into pET23-his<sub>6</sub>-SUMO vector in the same fashion. All constructs were sequenced after ligation and restriction analysis.

The nonprocessed *dnaX*<sub>np</sub> expression construct was generated by incorporating a -1/+1 nucleotide frameshift using overlapping PCR. First, amplification generated the -1, 5' end (OPC 280/348) and the +1, 3' end (OPC 349/281) individually from pET23-his<sub>6</sub>-SUMO-*dnaX*. Next, PCR using outer primers (OPC 280/281) combined with the previous individual amplifications as template produced an amplified, full-length *dnaX*<sub>np</sub> product. Full-length product was digested then ligated into pET23-his<sub>6</sub>-SUMO vector. pET23-his<sub>6</sub>-SUMO-*dnaX*<sub>np</sub> was sequenced to validate correct nucleotide deletion and addition.

Carboxy-terminal *ssrA* addition to DnaX 2-535 and DnaX was created through a two-step, extension PCR. Initial PCR amplification (OPC 280/533, 2-535; 280/537, 2-608) produced nucleotide sequences encoding for either DnaX 2-535 or DnaX with a portion of the *ssrA* tag. These inserts were then re-amplified (OPC 280/317) to extend and form a complete *ssrA* tag (protein-ANDNFAEEFAVAA-cooh).

His<sub>6</sub>-DHFR insert was amplified from a *E. coli* DHFR containing template plasmid (gift from L. Gierasch) using OPC 569/570. His<sub>6</sub>-SUMO was digested and removed from pET23-his<sub>6</sub>-SUMO vector using NheI/AgeI restriction sites. His<sub>6</sub>-DHFR was ligated into the same position creating pET23-his<sub>6</sub>-DHFR, a vector that appends his<sub>6</sub>-DHFR upstream to gene of interest. Both *dnaX* (317-608) and *dnaX* (436-608) were sequenced after digestion and ligation downstream of his<sub>6</sub>-DHFR.

#### **4.7.4 *Caulobacter* expression plasmids and strain construction.**

*Caulobacter* were grown in PYE rich media with standard antibiotic concentrations [20]. All *Caulobacter* expression plasmids append an N-terminal M2-FLAG tag that was used for detection with standard SDS-PAGE / Western Blotting protocols. Vanillate was used at 0.5 mM for induction in PYE [20], xylose was used at 0.2% in PYE (PYEX).

pENTR-dnaX was generated from cloning *dnaX* using restriction with AgeI/XhoI from pET23-his<sub>6</sub>-SUMO-*dnaX* into an ENTR vector generated from pENTR D/TOPO that contains unique AgeI/XhoI sites. The same strategy was used to generate pENTR-*dnaX* truncations and pENTR-*dnaXnp* from the appropriate expression plasmids. Primers designed for (OPC 590/591) site directed mutagenesis of pENTR-*dnaX* were 5' phosphorylated and used to generate pENTR-*dnax*-A175D DNA using Phusion-based mutagenesis. Inducible M2-FLAG tagged vectors suitable for expression in *Caulobacter* were generated via LR-recombination of pENTR constructs with appropriate pDEST vectors as before to generate low-copy (pLX and pVAN) and

medium-copy (pHX) vectors[20]. pHX-RR+15 expresses the receiver domain of CtrA+15 residue degradation and is used as a control expression plasmid for the pHX plasmid.

Plasmids capable of propagating in *Caulobacter* were transformed via electroporation into competent CB15N *C. crescentus* and selected for on appropriate media. Replacement of chromosomal *dnaX* alleles was performed by transforming pENTR-*dnaX*<sup>ts</sup> (DnaX-A175D) or pENTR-*dnaX*(1-462). Because pENTR plasmids cannot replicate in *Caulobacter*, antibiotic resistance can only be acquired if the plasmids integrate. Homologous recombination between the promoter-less plasmid *dnaX* sequence and chromosomal *dnaX* sequence results in only one expressed *dnaX* variant driven by the native promoter. Successful gene replacement was screened by PCR or by temperature sensitive phenotypes.

#### **4.7.5 ClpX depletion in *C. crescentus*.**

Depletion strain CPC26 (same as UJ200; NA1000 *clpX*::spec with *clpX* integrated at the *xylX* locus) was transformed with a low copy, vanillate inducible plasmid which expresses N-terminal M2FLAG tagged DnaX (pVan M2-*dnaX*) to generate CPC200). After growing overnight in PYEX plus antibiotics, the culture was back diluted to an OD600 of 0.1 in PYEX plus antibiotics. After reaching an OD600 of 0.3, the culture was centrifuged at 6000 g for 7 min and resuspended in PYE (no xylose) twice. Final pellet was resuspended into PYE with appropriate antibiotic, to original culture volume. Resulting culture was split into two equivalent volumes and supplemented with 0.2% xylose (to maintain ClpX expression) or glucose (to deplete ClpX). Both xylose and glucose containing cultures were further split into 2 equal volumes. Vanillate was immediately added to one set, and all cultures were allowed to grow for two hours at 30°C, at which point vanillate was added to the remaining set of cultures, and all cultures were allowed to grow an additional hour. This procedure produced four time courses,

with induction of M2-DnaX either continuous during ClpX depletion, or initiated after ClpX had already been depleted for two hours. Samples were taken for western blotting at 0, 1, 2, and 3hrs.

#### **4.7.6 Microscopy and UV irradiation.**

1ml of *Caulobacter* cells taken directly from log-phase experiments were applied onto 1% agarose pad mounted slides and viewed by phase-contrast using a AxioCam Cm1 (Zeiss) with 1000X magnification. Images were analyzed using ImageJ (NIH) where lengths of individual cells (n=150) were measured by taking multiple points along the central axis. Resulting length data was binned using KaleidaGraph. For the UV irradiation experiments, liquid cultures of the appropriate strains (n=10) were irradiated with 10,000 mJ/cm<sup>2</sup> in a Stratagene UV-Stratalinker 1800 (254 nm). Strains were recovered for 14 hours to fix mutations, serially plated on nonselective and rifampicin (50 mg/ml) containing media, and number of colonies were counted after three to four days of growth. Three replicates of these experiments were conducted on different days and relative fraction of rifampicin resistant colonies in the  $\tau_{\text{only}}/\gamma_{\text{only}}$  versus  $\tau_{\text{wildtype}}/\gamma_{\text{only}}$  strains were calculated for each replicate.

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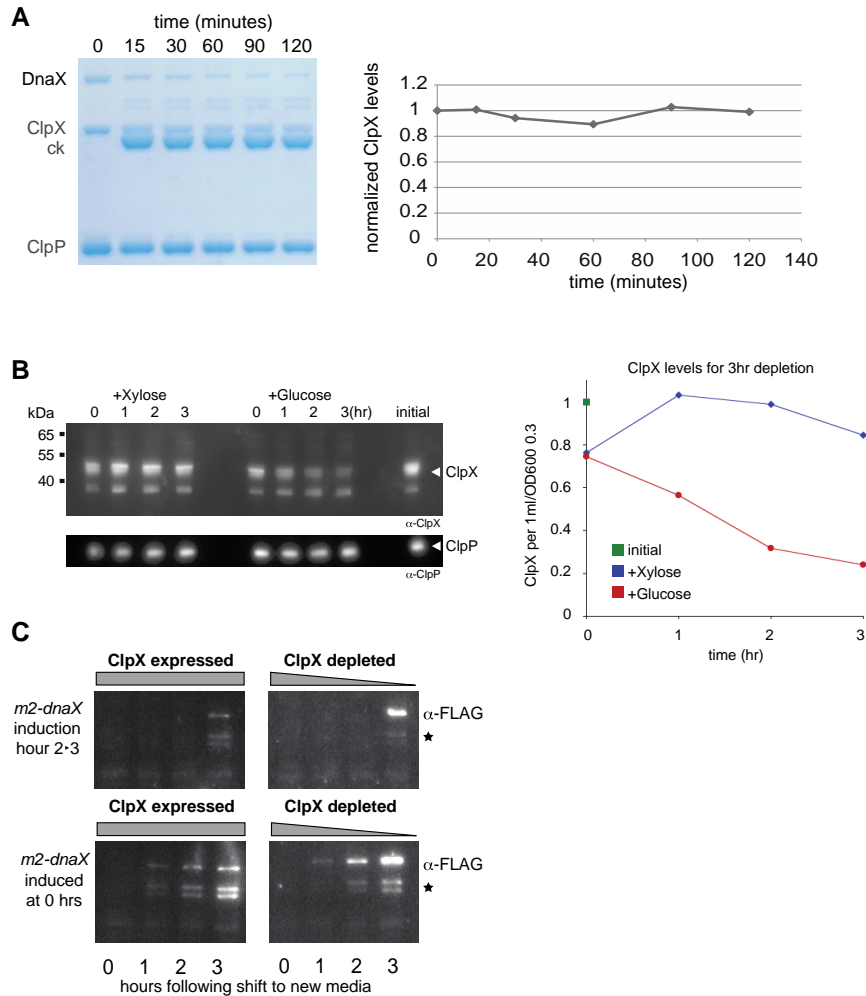
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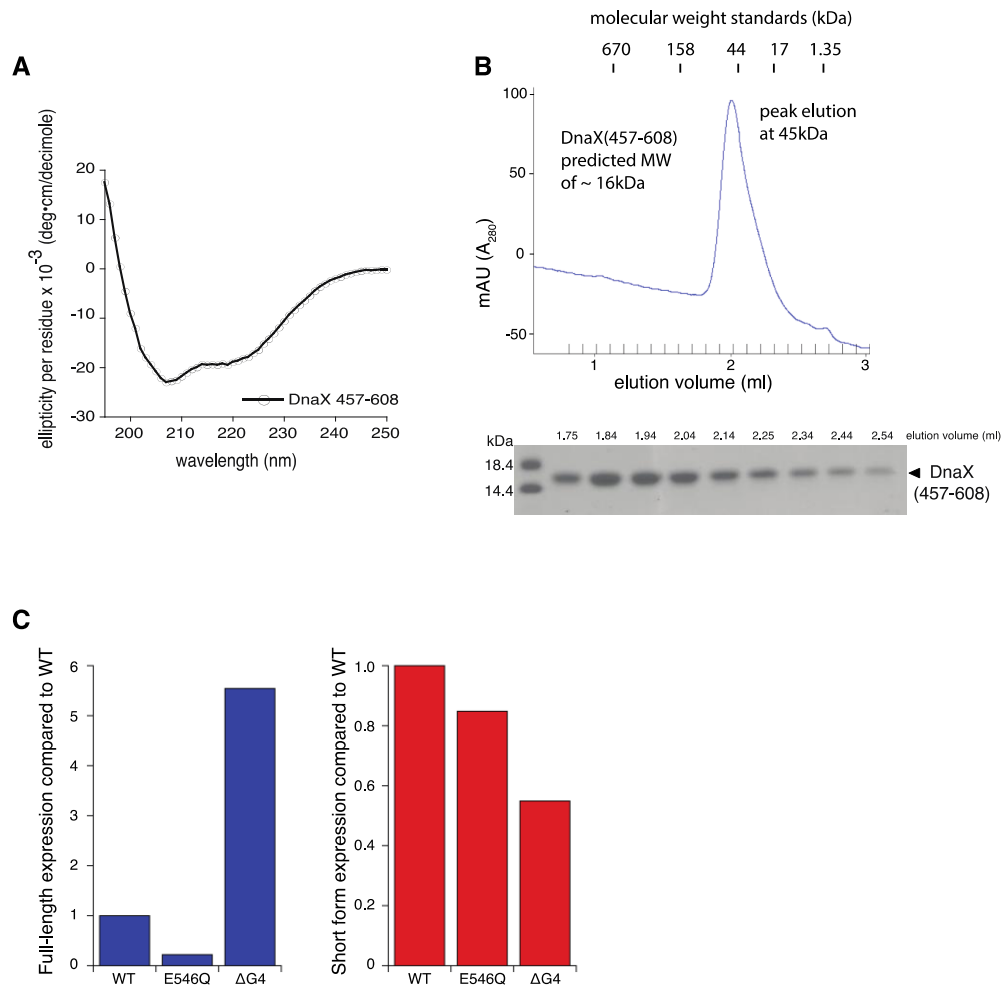
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## 4.9 Supplemental Information



### Supplemental 4.1 ClpP remains during ClpX depletion, DnaX loses processing

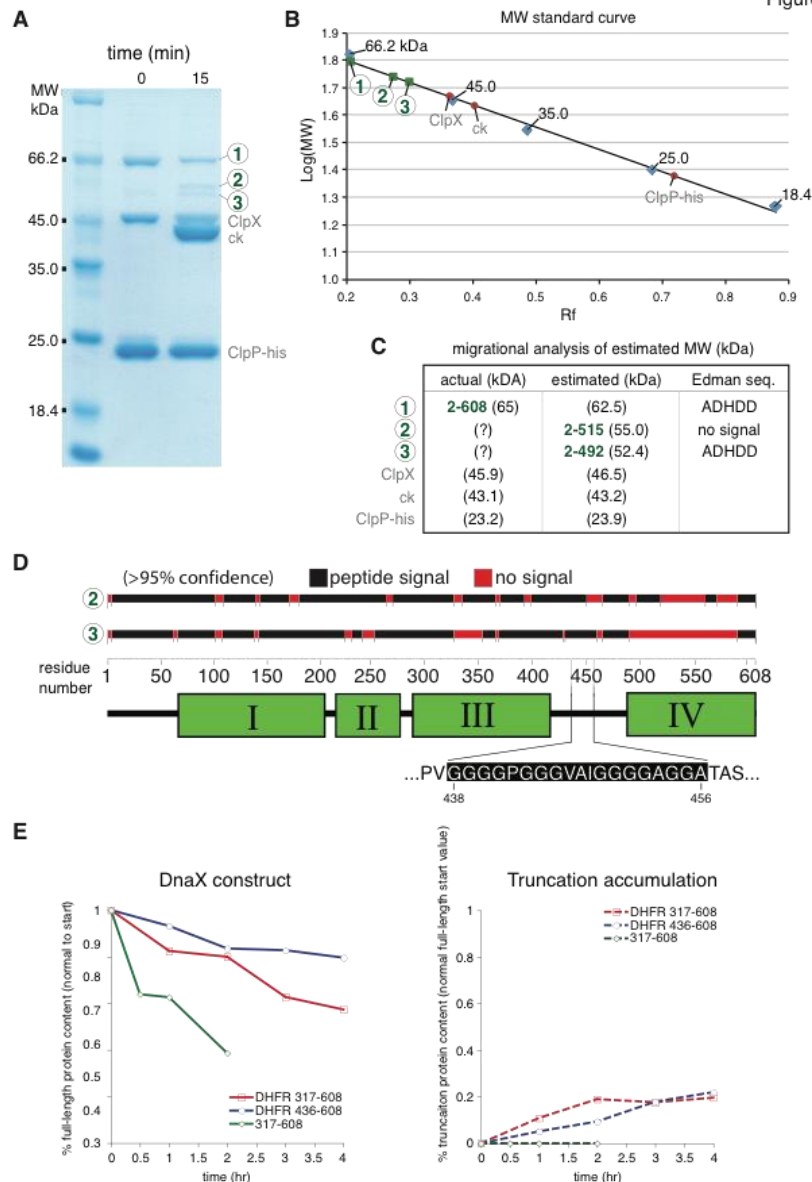
A. (in support of Figure 4.1B) Quantification of ClpX (relative to ClpP) shows that ClpX is not itself degraded during degradation of DnaX. B. Use of a ClpX depletion strain (see Methods) to determine effect of ClpXP on DnaX processing *in vivo*. The amount of ClpX that remains after 3 hours of depletion (glucose) is approximately 24% compared to levels prior to depletion. C. above: Depletion of ClpX as in B, but monitoring M2-DnaX upon one hour of induction after two hours of ClpX depletion had already occurred (i.e., monitoring newly synthesized DnaX). Under normal ClpX replete conditions, 27% of newly synthesized M2-DnaX is present as the full-length tau form. Following 3 hours of ClpX depletion, 60% of newly synthesized M2-DnaX is present as the full-length tau form. below: same as above, but M2-DnaX was induced immediately upon beginning ClpX depletion. Quantification of M2-DnaX levels and ClpX levels were performed using ImageJ.



## Supplemental 4.2 Mutations on the ordered DnaX C-terminal degron affect output of ClpXP degradation

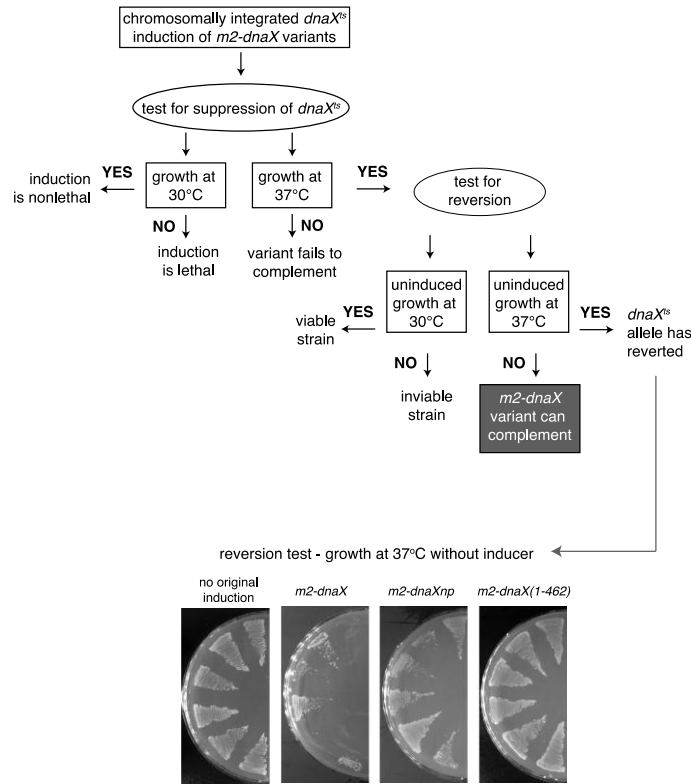
Circular dichroism (A) and gel-filtration (B) of the C-terminal residues 457-608 of DnaX shows that this protein domain is not unstructured. C. Quantification of M2-DnaX fragment composition from experiment shown in Figure 4.2C. Wild type *Caulobacter* transformed with vectors expressing either M2-dnaX, M2-dnaX E546Q, and M2-dnaX $\Delta$ 547-550 ( $\Delta$ G4) under xylose inducible control were grown to log phase and induced with 0.2% xylose for 3hrs. Quantifications were performed using ImageJ.

Figure S3



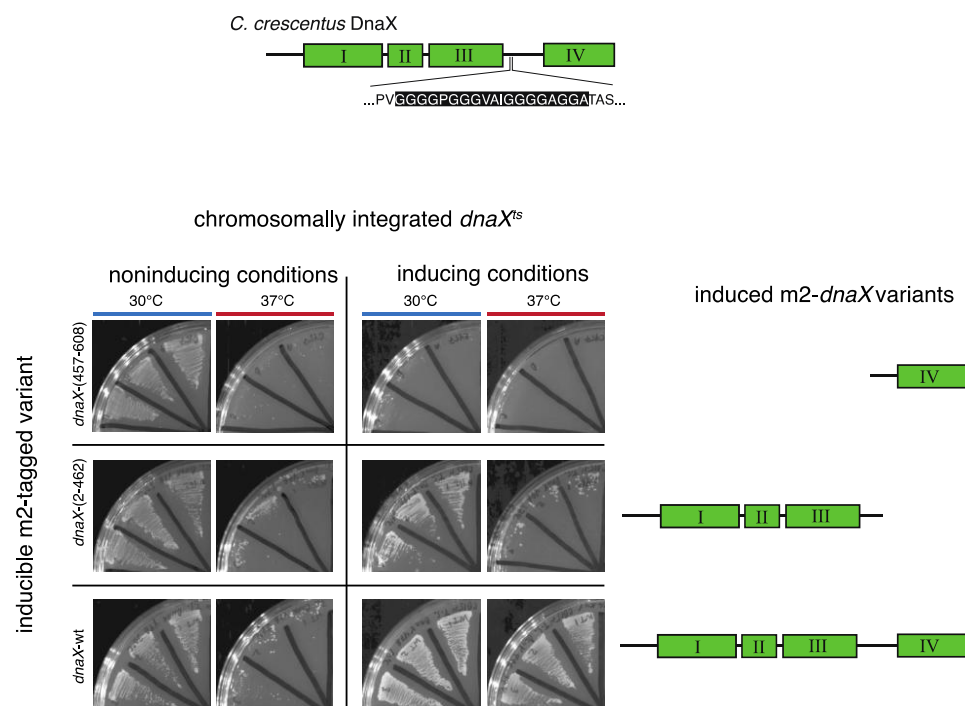
### Supplemental 4.3 DnaX gamma composition, size and accumulation

Characterization of DnaX truncation products generated *in vitro*. A. Illustration of representative gel, showing full-length (1) and two truncation fragments (2) and (3) of DnaX. B. Molecular weights of truncation products were estimated using known molecular weight ladder to generate a standard curve of migration distance versus molecular weight (MW) in kilodaltons on a semilog plot. MW markers, known protein components and DnaX isoforms are marked as shown. C. Table listing estimated MW (in kDa) of known proteins (ClpX, creatine kinase [ck], and his-tagged ClpP [ClpP-his]) and the actual MW based on protein sequence. Estimated MW of DnaX fragments were calculated based on protein sequence, assuming intact N-termini, and used to determine the polypeptide length (in green) consistent with those estimated MW. Also shown are results of Edman degradation/sequencing of the full-length and smallest fragment, showing that the N-terminus of the smallest fragment is the same as that of the full length (consistent with the native N-terminus). D. Truncations labeled (2) and (3) were also subjected to trypsinization/mass spectrometry. Cartoon is a depiction of regions of the DnaX polypeptide that contain at least one peptide identified with > 95% confidence by mass spectrometry (in black) and regions where no high confidence peptides were found (in red). E. Quantification of DHFR-DnaX variants from Figure 4.3B-D. left: Quantification of the full-length protein for each variant. right: Quantification of truncation product buildup for each variant. All quantifications were performed using ImageJ.



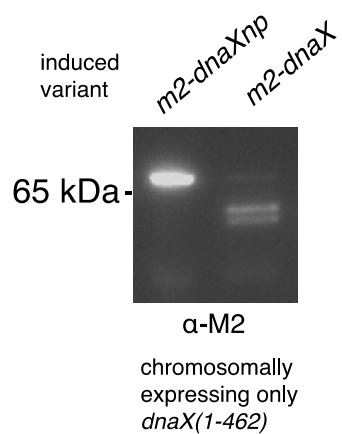
#### Supplemental 4.4 *dnaX<sup>ts</sup>* complementation decision tree

Reversion analysis to validate complementation of *dnaX<sup>ts</sup>* by plasmids expressing either *m2*-tagged wildtype *dnaX*, *dnaXnp* ( $\tau$ -only), or *dnaX(2-462)* ( $\gamma$ -only) at nonpermissive conditions. The flowchart depicts the steps taken to distinguish if colonies growing in nonpermissive, inducing conditions are a result of complementation or a reversion of the temperature sensitive phenotype. Shown at the bottom are representative colonies of strains expressing the labeled constructs that originally grew on induction media at 37°C, then were restreaked on noninducing media at 37°C to assess if they had lost the temperature sensitive phenotype. Colonies arising from cells expressing wildtype *m2-dnaX* generally fail to grow subsequently at 37°C in noninducing conditions. Thus, these cells have retained their temperature sensitivity and we infer that the original growth was a result of the ability of *m2-dnaX* to complement the temperature sensitive *dnaX<sup>ts</sup>* allele. In contrast, colonies arising from cells that originally did not express any additional *dnaX* variant ("no original induction") consistently grow well upon restreaking onto noninducing media at 37°C. We therefore infer that the original growth of these colonies was due to loss of the temperature sensitive phenotype. Colonies of cells originally expressing *m2-dnaXnp* or *m2-dnaX(2-462)* grow better than wildtype *m2-dnaX* strains upon subsequent passage at 37°C in noninducing conditions. We interpret this to mean that the delayed colony growth originally seen during inducing, nonpermissive conditions (main Figure 4.4) originally arose principally through reversion of the temperature sensitive phenotype, rather than complementation of *dnaX<sup>ts</sup>*.



#### Supplemental 4.5 *dnaX<sup>ts</sup>* complementation by full-length, domains I-III or IV fragment

*dnaX<sup>ts</sup>* can be complemented by wildtype *dnaX* expression, but not expression of either N-terminal (residues 2-462;  $\gamma$ -only) DnaX constructs nor by C-terminal DnaX constructs (residues 457-608). See Figure S4.4 for details of testing reversion. Note that expression of the C-terminal domain of DnaX alone is toxic to cells, even in permissive temperature conditions.



#### Supplemental 4.6 Validation of *m2-dnaXnp* and *m2-dnaX* expression

Cells expressing  $\gamma$ -only DnaX from the chromosome fail to grow in the absence of expression of full length DnaX, but induction of either *m2-dnaX* or *m2-dnaXnp* results in robust growth (see Figure 4.4 main text). Western blot analysis showing that growth of colonies from these cells inducing *m2-dnaXnp* do not arise from an unanticipated processing of the M2-DnaXnp protein.

**Table 4.1 Oligonucleotide list for this chapter**

OPC #	sequence
280	CACCGCTaccggtggtGCCGACCACGACGACCTCTC
281	aagcttCTCGAGttaGCCCTCTTCCTCGTCCG
309	CACCGCTaccggtggtGCCGGCAAGACCAAGGACGC
310	aagcttCTCGAGttaGtCgTCTTCCTCGTCCGGCTC
317	AAGCTTctcgagTTAagctgcaacagcgaattcttctcg
348	ccgccgatcgcaacgccaccgcccggaccgccaccaccaAGGCGCGCCGTCGCGCAG
349	cgggtggcggttcgatcgccggcggtggcgcgggcgggcgGACGGCCTCGGCCAGTCT
350	CACCGCTaccggtggtACGGCCTCGGCCAGTCT
361	aagcttCTCGAGttaAGACTGGGCCGAGGCCGT
363	aagcttCTCGAGttaGGCCGTCTCGGGGGTGAG
366	AATATActcgagTCAGTCCTGCTTGATGGCCTC
367	AATATActcgagTCAAGCCAGCACCGGCAT
529	AATATActcgagTTCCTTCAGGAAGCGAAC
530	AATATActcgagTTCCTTCAGGAAGCGAAC
533	agcgaattcttctgcgaagttgtcgtagcCTCCTTCAGGAAGCGAACAAAGG
537	agcgaattcttctgcgaagttgtcgtagcGCCCTCTTCCTCGTCCG
569	AATTAAgctagccatcatcatcatcatATCAGTCTGATTGCGGCGTTA
570	TATATAaccggtCCGCCGCTCCAGAATC
590	gatCGCTACAAGGTCTACATCATCGAC
591	CTCGACCGGGGCGTAGCG
592	TATATAaccggtCCTGTGGGTGGTGGCG



**Table 4.2 *E. coli* strain list for this chapter**

n.b. JK10 is BL21DE3  $\Delta$ ClpP.

EPC #	strain	resistance
524	BL21(DE3) pET23-his <sub>6</sub> -SUMO-dnaX	AMP
525	BL21(DE3) pET23-his <sub>6</sub> -SUMO-dnaX-2-462 (NTD)	AMP
526	BL21(DE3) pET23-his <sub>6</sub> -SUMO-dnaX-2-535	AMP
527	BL21(DE3) pET23-his <sub>6</sub> -SUMO-dnaX-2-556	AMP
530	BL21(DE3) pET23-his <sub>6</sub> -SUMO-dnaX-DD	AMP
531	BL21(DE3) pET23-his <sub>6</sub> -SUMO-dnaX-457-608 (CTD)	AMP
532	BL21(DE3) pET23-his <sub>6</sub> -SUMO-dnaX-np	AMP
535	BL21(DE3) pET23-his <sub>6</sub> -DHFR-dnaX-317-608	AMP
536	BL21(DE3) pET23-his <sub>6</sub> -DHFR-dnaX-436-608	AMP
537	JK10 pET23-his <sub>6</sub> -SUMO-dnaXssrA	AMP
551	TOP10 pENTR-dnaX	KAN
552	TOP10 pENTR-dnaX-2-462 (NTD)	KAN
553	TOP10 pENTR-dnaX-457-608 (CTD)	KAN
554	TOP10 pENTR-dnaXnp	KAN
555	TOP10 pLX M2-dnaX	TET
556	TOP10 pLX M2-dnaX 2-462 (NTD)	TET
557	TOP10 pLX M2-dnaX 457-608 (CTD)	TET
558	TOP10 pLX M2-dnaXnp	TET
561	TOP10 pHX M2-dnaX	SPEC/strep
563	TOP10 pVAN M2-dnaX	KAN

**Table 4.3 *Caulobacter crescentus* strain list for this chapter**

n.b. The parent of CPC200 is UJ200 (Jenal U & Fuchs T (1998) *An essential protease involved in bacterial cell-cycle control*. EMBO J 17(19):5658-5669.)

CPC #	strain	resistance
176	CB15N wildtype	none
177	CB15N pLX M2-dnaX	TET
180	CB15N pLX M2-dnaXnp	TET
183	CB15N pHX M2-dnaX	SPEC/strep
185	CB15N dnaX::dnaX(2-462) pLX M2-dnaX	KAN/TET
187	CB15N dnaX::dnaX(2-462) pLX M2-dnaXnp	KAN/TET
191	CB15N dnaX::dnaX <sup>ts</sup>	KAN
192	CPC191 pLX M2-dnaX	KAN/TET
193	CPC191 pLX M2-dnaX-2-462 (NTD)	KAN/TET
194	CPC191 pLX M2-dnaX-457-608 (CTD)	KAN/TET
195	CPC191 pLX M2-dnaX-np	KAN/TET
196	CPC191 pLX M2-dnaX-np pHX M2-dnaX	KAN/TET/SPEC
197	CPC191 pLX M2-dnaX-np pHX M2-dnaX-2-462 (NTD)	KAN/TET/SPEC
199	CPC191 pLX M2-dnaX-np pHX-RR+15	KAN/TET/SPEC
200	NA1000 ClpX::Spec ,xylX:ClpX ,pVAN M2-dnaX	SPEC/TET/KAN

## 5. THE ESSENTIAL ROLE OF CLPXP IN *CAULOBACTER CRESCENTUS* REQUIRES SPECIES CONSTRAINED SUBSTRATE SPECIFICITY

*as written by Vass R.H., Nascembeni J., and Chien P.,*

*Frontiers in Molecular Biosciences 2017*

### General Chapter Overview

Chapter 5 investigates the relationships drawn between different modes of ClpX recognition and the effects that recognition has on *Caulobacter* cells. Here, recognition based changes categorized by differences contained in the lesser-conserved stretches found between higher domain conservation ultimately affect cell physiology and efficiency in substrate pool degradation. The varying conservation surrounding more conserved regions core domains is shown to exhibit a cumulative effect in shaping overall cell growth characteristics. Interestingly, amidst the physical differences found between *Caulobacter crescentus* and *E. coli* ClpX orthologs, some substrate and adaptor use is conserved suggesting an underlying common need for similar systems to also be regulated in a similar fashion. Here, unique protein interactions require the cognate protease found in that organism for proper protein homeostasis, especially for direct recognized ClpX target DnaX in *Caulobacter*.

My major contributions to this chapter centers on performing all of the biochemistry and molecular biology experiments and generating figures required for this work, conceptual and experimental design, writing and mentoring this work from start to submission.

## 5.1 Abstract

The ClpXP protease is a highly conserved AAA+ degradation machine that is present throughout bacteria and in eukaryotic organelles. ClpXP is essential in some bacteria, such as *Caulobacter crescentus*, but dispensable in others, such as *Escherichia coli*. In *Caulobacter*, ClpXP normally degrades the SocB toxin and increased levels of SocB result in cell death. ClpX can be deleted in cells lacking this toxin, but these  $\Delta clpX$  strains are still profoundly deficient in morphology and growth supporting the existence of additional important functions for ClpXP. In this work, we characterize aspects of ClpX crucial for its cellular function. Specifically, we show that although the *E. coli* ClpX functions with the *Caulobacter* ClpP *in vitro*, this variant cannot complement wildtype activity *in vivo*. Chimeric studies suggest that the N-terminal domain of ClpX plays a crucial, species-specific role in maintaining normal growth. We find that one defect of *Caulobacter* lacking the proper species of ClpX is the failure to properly proteolytically process the replication clamp loader subunit DnaX. Consistent with this, growth of  $\Delta clpX$  cells is improved upon expression of a shortened form of DnaX *in trans*. This work reveals that a broadly conserved protease can acquire highly specific functions in different species and further reinforces the critical nature of the N-domain of ClpX in substrate choice.

## 5.2 Introduction

Energy dependent proteolysis is a cellular process that maintains protein homeostasis, quality control, and allows for temporal changes in protein concentration required for cell signaling [1]. ClpXP is a conserved protease complex that performs highly targeted degradation. ClpXP is a two-part protease system consisting of a regulatory element (ClpX) and peptidase (ClpP) and is present throughout biological systems, ranging from bacteria to eukaryotic organelles. ClpX requires the use of ATP to self oligomerize, recognize, and unfold target proteins. The unfoldase has two main functions; 1) recognize substrates and 2) translocate them into the ClpP pore for degradation. The AAA+ domain of ClpX contains the Walker motifs that bind/hydrolyze ATP and the central pore loops required for substrate engagement [2]. An additional unique feature of ClpX is its N-domain, which is needed for recognition of some protease substrates. Regardless of how they are recognized, all substrates must be translocated to ClpP. Therefore, ClpX must interact effectively with ClpP to realize the full potential of this protease [3,4].

The ClpX unfoldase must regulate which substrates are targeted for destruction by the ClpP chamber [2]. For example, in the bacterium *Caulobacter crescentus*, ClpX activity responds to cell cycle cues and stresses to meet the proteolytic demands as needed [5-10]. To accomplish these different proteolytic tasks, ClpXP recognizes substrates using both simple degradation tags (degrons) and with the assistance of adaptor proteins that promote degradation of new substrate pools in a ClpX N-domain dependent manner [2,8]. One instance of this complex regulation is during trans-translation, where the rescue of stalled ribosomes is accompanied by the appending of the SsrA peptide, which is recognized by the ClpXP protease, to improperly translated polypeptides leading to their destruction [11-13]. Although this base recognition is independent of the ClpX N-domain, the SspB adaptor can further improve degradation of SsrA-tagged substrates by binding the N-domain of ClpX [14].

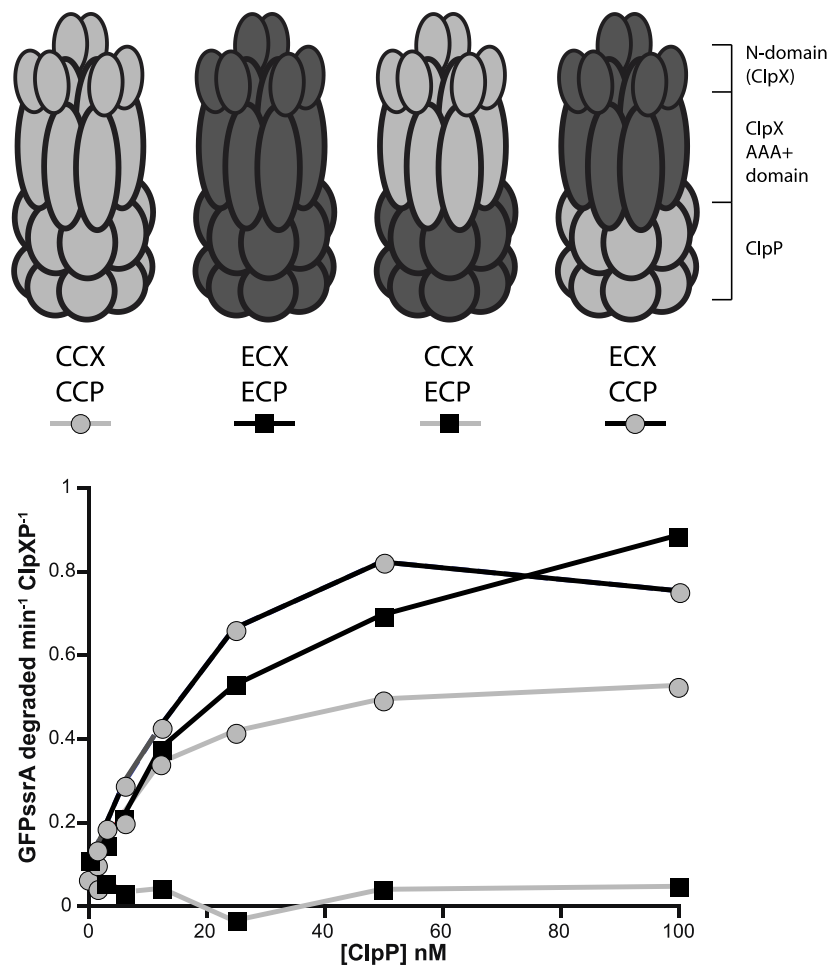
The ClpXP complex is not essential in all organisms. For example, ClpXP is dispensable in *E. coli* [15], but is required in *Caulobacter crescentus* [5,16]. Recent work points to a critical role of ClpXP in *Caulobacter* through the essential processing of the replication clamp loader subunit DnaX, driving cell cycle progression, and destruction of the toxin SocB - processes that are absent in *E. coli* [17-19]. Interestingly, despite high homology, the *E. coli* ClpX cannot complement the essential ClpX function in *Caulobacter* cells [5,16]. Here, we use chimeric variants of ClpX to determine which features of this protease are important for either species-specific or species-nonspecific activity. We find that the N-domain of ClpX plays an especially important role in regulating essentiality in *Caulobacter*, but that expression of a non-complementing ClpX provides benefit during cell growth. Together, our work demonstrates how ClpXP specificity regulates species-specific responses in a bacterium where this protease is essential.

### 5.3 Results

#### 5.3.1 *E. coli* ClpX forms an active protease with *Caulobacter* ClpP *in vitro*

Prior work suggests that the *E. coli* ClpX cannot substitute for ClpX in *Caulobacter* [16]. What are the differences between *E. coli* ClpX (ECX) and *Caulobacter* ClpX (CCX) that restrict essentiality in *Caulobacter*? An alignment of ECX to CCX protein sequences reveals high identity (68%) and a total homology of ~90% (Figure S5.1). We sought to understand why these enzymes do not substitute for each other despite their high similarity. A simple explanation for the inability of ECX to complement in *Caulobacter* may be an inability for ECX to bind with the *Caulobacter* ClpP and form an active protease. We tested this hypothesis by monitoring ClpXP dependent degradation of GFP-ssrA where loss of fluorescence occurs when ClpX successfully delivers substrate to ClpP (Figure 5.1).

Both ECX and CCX are able to deliver substrate to *Caulobacter* ClpP (CCP), while only ECX can recognize and degrade GFPssrA together with *E. coli* ClpP (ECP, Figure 5.1). By titrating ClpP, we can derive an effective binding of ClpX to ClpP as a measure of protease activation ( $K_{activation}$ ) and find similar strengths of interactions between ClpX and ClpP in those combinations that result in an active protease (Table 5.1). This suggests that both ECX and CCX associate similarly with CCP. Note that the CCX + ECP combination fails to degrade GFPssrA, (Figure 5.1), but because this combination is not germane to this current work, we did not further explore this observation in this manuscript. Our major conclusion from this characterization is that it seems that ECX forms a productive protease with CCP, therefore the failure of ECX to replace CCX *in vivo* [16], likely stems from a failure to maintain a particular substrate degradation profile rather than a failure of protease assembly. We decided to capitalize on this difference in activity to explore how species-specific elements of ClpX are required in different bacteria.



**Figure 5.1** *E. coli* ClpX forms an active protease with *Caulobacter crescentus* ClpP.

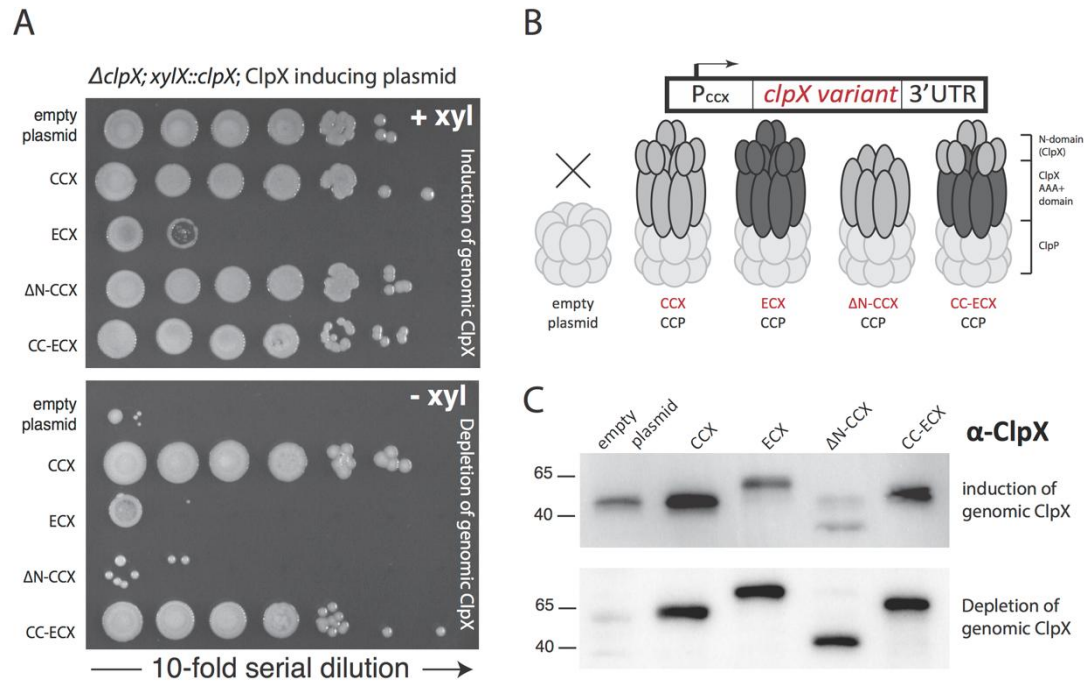
Degradation reactions of 1  $\mu$ M GFP-ssrA by 50 nM ClpX<sub>6</sub> with varying concentrations of ClpP<sub>14</sub> as shown on the x-axis. Initial rates of degradations are plotted as a function of ClpP<sub>14</sub> concentration.



### 5.3.2 The N-domain of *Caulobacter* ClpX harbors an essential species-specific function

Although the ClpX pore is critical for substrate recognition, the ClpX N-domain provides additional specificity, often driven upon the binding of the N-domain by adaptor proteins that aid in degradation of substrates. We speculated that the ClpX N-domain contains species-specific motifs that provide for the essential activity in *Caulobacter*. Because ECX could form an active protease with CCP *in vitro*, we inferred that the AAA+ domain of ECX was sufficient to interact with CCP, as the N-domain is dispensable for the ClpX-ClpP interaction [3]. Therefore, we used this system to determine how different variants and chimeras of ECX or CCX could support viability in *Caulobacter*.

We expressed different ClpX variants in a strain background where the endogenous ClpX could be depleted [16]. Similar to what had been reported previously [16], expression of ECX from a plasmid failed to complement, while similar expression of CCX restored growth (Figure 5.2A). Expression of a CCX lacking the N-domain ( $\Delta$ N-CCX) was also unable to support viability (Figure 5.2A) [20]. Interestingly, a chimeric construct consisting of the N-domain of CCX fused to the AAA+ domain of ECX (CC-ECX) was able to restore viability in this background (Figure 5.2A,B). Western analysis confirms the expression of the appropriate constructs and the depletion of the endogenous ClpX (Figure 5.2C). The presence of ECX also affects normal *Caulobacter* growth even in the presence of CCX (Figure 5.2A; +xyl), which we speculate may be due to ECX binding to CCP and disrupting the formation of productive CCX+CCP complexes. Taken together with our *in vitro* work (Figure 5.1), our data suggests that the CCX N-domain is required for identification of substrates and proper degradation, which is ultimately needed for *Caulobacter* survival.



**Figure 5.2 Only ClpX that contains the *Caulobacter* N-domain is able to support viability.**

(A) Depletion of genomic ClpX by removal of xylose provides a background to test if plasmid encoded, constitutively expressed ClpX variants are capable of complementing viability. Survival seems restricted to constructs that contain the *Caulobacter* N-domain (see Supplemental Figure S5.3 for replicate). (B) Plasmid constructs contain the *Caulobacter* ClpX promoter to drive constitutive expression of the ClpX variants. (C) Monitoring of ClpX levels by Western illustrates the successful depletion of genomic ClpX and the presence of plasmid expressed ClpX variants.

### 5.3.3 Bypassing the essential requirement for ClpX reveals nonessential proteolysis important for growth

Recent work suggests that the regulated destruction of the SocB toxin by the ClpXP protease via the adaptor SocA justifies the essential need for ClpX in *Caulobacter* [17]. In this model, depletion of ClpXP results in accumulation of the SocB toxin and cell death. It is possible that the CCX N-domain contains unique regions needed for interacting with the SocA adaptor to promote SocB degradation. If so, these regions are either absent in ECX or they are masked, which would explain the finding that ECX fails to complement viability (Figure 5.2A). An alternative model is that the ECX engages inappropriately with other target proteins, which results in cell death due to prolific degradation. We sought to distinguish between these models by taking advantage of strains where *socB* is deleted.

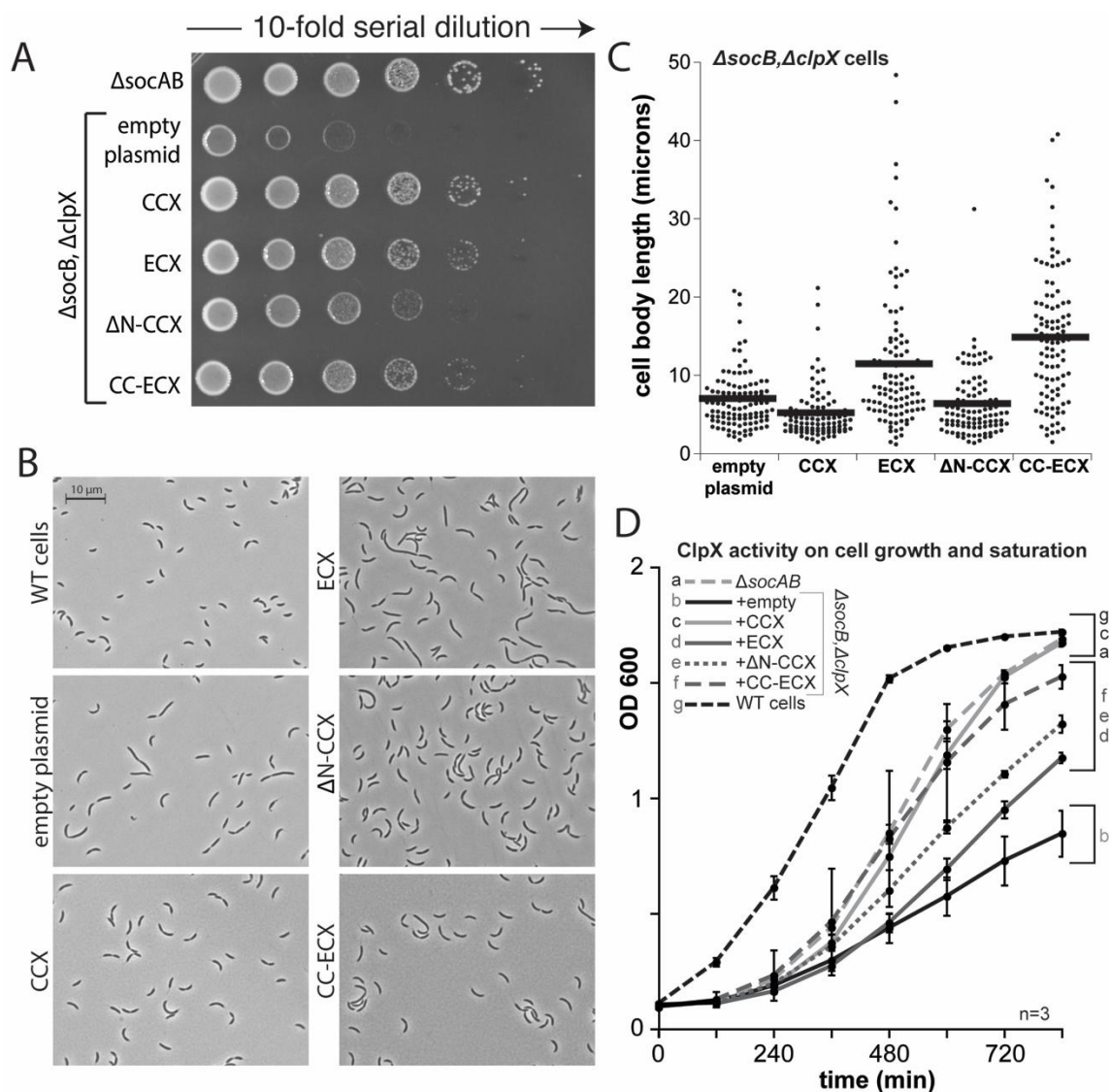
In cells lacking SocB, *clpX* could be deleted, but these cells are abnormal and show poor viability upon plating (Figure 5.3A). As expected, expression of CCX restored viability in a dilution-plating assay (Figure 5.3A). However, in contrast to prior observations ([16], Figure 5.2A), expression of ECX complements growth (Figure 5.3A,D). The  $\Delta$ N-CCX construct also improves viability, though less effectively than variants of ClpX with an N-domain (Figure 5.3A,D). Microscopy studies reveal that expression of CCX in  $\Delta clpX \Delta socB$  cells restores normal morphology and cell length (Figure 3B,C). Interestingly, although expression of ECX restores viability, cell morphology and cell length are still dramatically perturbed (Figure 5.3B,C). This perturbation is also seen with expression of the chimeric CC-ECX construct (Figure 5.3B,C), suggesting that species-specific differences in the ClpX AAA+ domain are responsible for these changes in cell morphology. Consistent with this interpretation, expression of the  $\Delta$ N-CCX restores cell length more fully than either of the constructs containing the ECX AAA+ domain (Figure 5.3B,C). Thus, it seems that there are species-specific N-domain dependent and AAA+

domain-dependent substrate recognition profiles that both contribute to the role of ClpX in *Caulobacter*.

#### **5.3.4 Species-specific processing of DnaX is needed for robust growth**

Given the species-specific nature of the phenotypic complementation, we next explored the molecular consequences of ClpX variant expression.

DnaX is a subunit of the replication clamp loader complex that is responsible for sliding clamp dynamics during replication and DNA damage responses [21]. In *Caulobacter*, full length DnaX (also called  $\tau$ ) is processed by the ClpXP protease to generate shorter fragments ( $\gamma 1$  and  $\gamma 2$ ) that are critical for survival and a robust DNA damage response (Figure 5.4A) [18]. Because  $\Delta socB$  cells can tolerate the loss of ClpX, we examined the levels of DnaX in this background. In line with our expectations, DnaX was not processed in cells lacking ClpX (Figure 5.4B). Previous *in vitro* work suggested that the N-terminal domain of ClpX plays an essential role for proteolytic recognition of DnaX [18] and, consistent with this model, cells expressing  $\Delta N$ -CCX fail to process DnaX. However, this N-domain dependence is species-specific, as cells expressing ECX also do not correctly process DnaX, resulting in loss of the shortest ( $\gamma 2$ ) DnaX and accumulation of full-length DnaX (Figure 5.4B). The ECX AAA+ domain is able to process DnaX correctly as expression of the CC-ECX chimeric ClpX, which contains the ECX AAA+ domain, is sufficient to restore the production of both normal DnaX



**Figure 5.3 The presence of ClpX shapes normal growth in *Caulobacter*.**

(A) The presence of any ClpX variant improves growth when both SocB and ClpX are absent (see Supplemental Figure S5.3 for replicate). (B) Microscopic examination shows differences in cell length and morphology dependent on ClpX variant. (C) Quantification of cell length (in microns) for strains shown in B,  $n > 100$ . (black bars denote mean length in microns for each strain). (D) Expression of any ClpX improves cell mass accumulation ( $n=3$ , error bars are standard deviation). Restoration of wildtype growth requires both the *Caulobacter* N-domain and AAA+ domain (a,c), but expression of any ClpX variant results in partial growth restoration (d,e,f) compared to no ClpX (b).

fragments. Therefore, species-specific combinations of the N-domain and the ClpX AAA+ domain are needed for normal processing and degradation of DnaX.

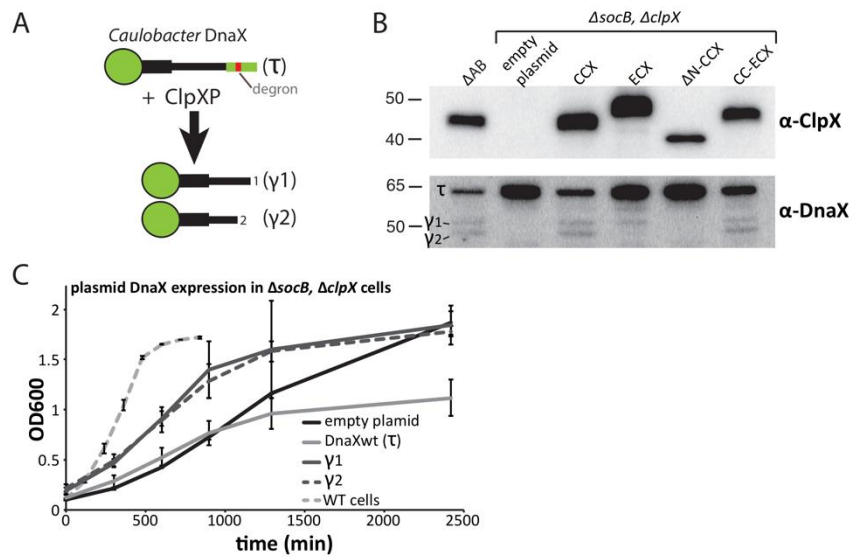
Previously, we showed that DnaX processing is essential for wildtype growth [18], however  $\Delta socB \Delta clpX$  strains are viable even though DnaX is not processed in this background (Figure 5.4B). Given the sickness of these cells, we asked if expression of the  $\gamma$ -fragments of DnaX could improve growth in these strains. Consistent with a critical need for DnaX isoforms, we found expression of either  $\gamma 1$  or  $\gamma 2$  DnaX increased growth rate in liquid cultures, compared to the empty plasmid control (Figure 5.4C). Curiously, expression of full length DnaX (which only generates  $\tau$  in this ClpX-free strain) inhibits growth and reduces density at saturation suggesting that an excess of  $\tau$  is toxic. Despite the clear improvement in growth, the doubling time of  $\gamma 1$  or  $\gamma 2$  expressing strains is still ~9-10 hours (Figure 5.4C), substantially longer than the ~90 minute doubling time of wildtype *Caulobacter* in these conditions. Therefore, there must be additional non-essential aspects of ClpXP degradation that promote normal robust growth.

### 5.3.5 Cell cycle adaptors do not rely on species restricted interactions with ClpXP

*Caulobacter* growth and development relies on adaptors that interact with the ClpX N-domain [6,10,17]. The ECX AAA+ domain is active (Figure 5.1) but the ECX variant results in a DnaX distribution different from CCX (Figure 5.4B). Therefore, we next asked if adaptor mediated degradation was altered in strains expressing ECX.

CtrA is a master regulator and replication inhibitor in *Caulobacter* that must be degraded during the transition from the swarmer to stalked cell to promote replication and developmental changes [5,22]. Degradation of the CtrA protein is an excellent model for N-domain dependent delivery as this process requires a multi-adaptor hierarchy consisting of CpdR, PopA and RcdA [6,9,10,23]. By monitoring the adaptor-dependent delivery of CtrA we could explicitly test if the

ECX N-domain was capable of supporting these adaptor interactions. As a read out of CtrA degradation, we used Western blotting to monitor levels of CtrA following inhibition of protein synthesis upon addition of chloramphenicol. As anticipated, cells containing the CCX N-domain (CCX, CC-ECX) can degrade CtrA while cells without ClpX or expressing  $\Delta$ N-CCX are unable to degrade CtrA robustly (Figure 5.5A). Cells expressing ECX as the only ClpX variant exhibit CtrA degradation similar to wildtype (Figure 5.5B).



**Figure 5.4 Processing of DnaX improves *Caulobacter* growth.**

(A) Processing DnaX  $\tau$  into either  $\gamma_1$  or  $\gamma_2$  requires ClpXP recognition of the degron and release of stable fragments. (B) Cells that lack ClpX contain only full length DnaX. Expression of ClpX variants harboring the *Caulobacter* N-domain result in processing of DnaX. Expression of ECX result in aberrant fragment formation while expression of  $\Delta$ N-ClpX fails to produce DnaX fragments (See Figure S5.4 for replicate blots). (C) In cells that lack both SocB and ClpX, supply of either  $\gamma_1$  or  $\gamma_2$  *in trans* increases the *Caulobacter* growth. Additional expression of wildtype DnaX results in a lower cell mass at saturation (n=3; error bars represent standard deviation).

Thus, the N-domain of ECX is able to support degradation through the adaptor hierarchy found in *Caulobacter*.

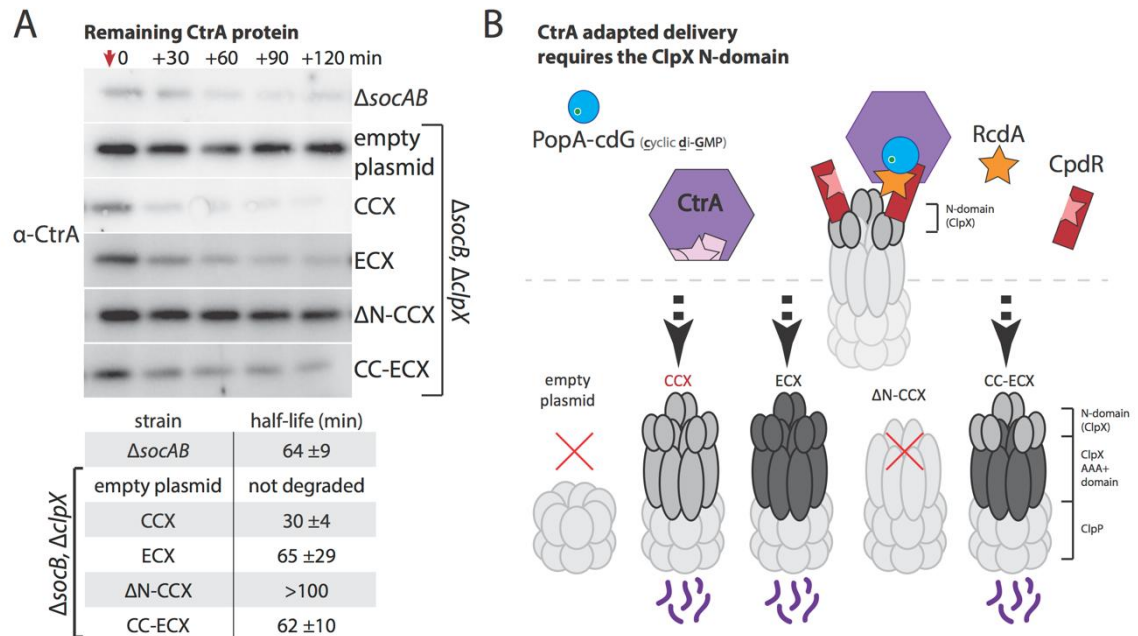
Our working model is that ECX fails to degrade the SocB toxin because the N-domain of ECX fails to bind the SocA adaptor (Figure 5.2). However, the N-domain of ECX appears fully competent to interact with the cell cycle adaptor hierarchy (Figure 5.5). Because adaptor-dependent delivery requires unique interactions supplied by the N-domain and contacts with the ClpX AAA+ domain, our work reveals a complexity in this regulation that results in both species-specific and species-nonspecific recognition of protease substrates.

## 5.4 Discussion

The presence of the ClpX unfoldase in all bacteria is likely due to a need for its protease activity. Given the similarity between orthologs, it is perhaps not surprising that many species of ClpXP can universally recognize some substrates based on conserved sequence or structural degons, such as SsrA-tagged proteins. Increasing the versatility of ClpX activity therefore requires additional elaboration of ClpX-substrate interactions. Adaptors can fill this role, but are not the only method of diversifying substrate recognition.

Our comparison of *E. coli* and *Caulobacter* ClpX reinforces the working model that the most conserved regions of the ClpX AAA+ domain support functions required for all protease activity, such as ATP hydrolysis, oligomerization and ClpP binding (Figure 6). More diverse regions appear to be the origin of species-specific activity. For example, both ECX and CCX contain the "IGF" motifs required for ClpP binding, but the area





**Figure 5.5** *E. coli* ClpX can use *Caulobacter* adaptors to effectively degrade CtrA.

(A) Measuring CtrA levels after addition of chloramphenicol (at red arrow) reveals that both CCX and ECX N-domains can support CtrA proteolysis. The table includes half-lives and standard deviation averaged over three individual experiments (see Supplemental Figure S5.5 for replicate blots and quantification). As expected, cells lacking ClpX or expressing only ΔN-ClpX fail to degrade CtrA [6]. (B) Model showing how rapid degradation of CtrA requires the combination of CpdR, RcdA, and PopA with cdG (cyclic di-GMP) to interact with the ClpX N-domain.

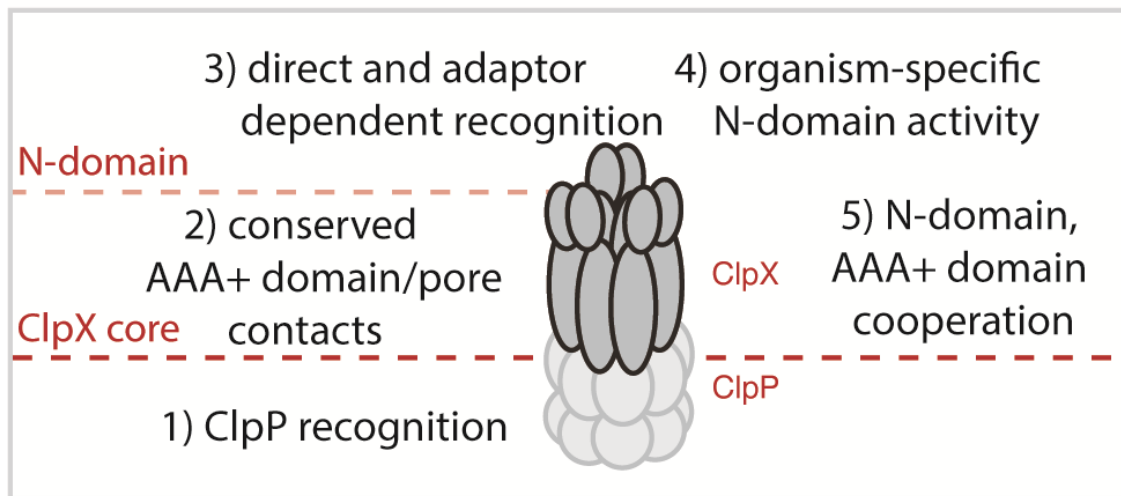
surrounding this region varies (Figure S5.1 and S5.2). This difference may explain the inability of CCX to interact with ECP in an *in vitro* setting. By contrast, the *Caulobacter* ClpX N-domain appears to support essential contacts required for *Caulobacter* viability that the *E. coli* N-domain does not provide. These contacts may include stringent recognition of substrates or interactions with critical adaptors needed for viability. We speculate that the differences in sequences

between these species of N-domains (Figure S5.1 and S5.2) may underlie these different binding profiles.

The N-domain alters substrate targeting to ClpX by directly recognizing substrates or cooperating with a diverse set of adaptors for target degradation. In our study, we find fusing the *Caulobacter*'s ClpX N-domain onto the AAA+ domain of *E. coli* ClpX restores the essential nature of ClpX in *Caulobacter*. We interpret this as evidence for the N-domain of the *Caulobacter* ClpX playing a unique role, such as facilitating degradation of the SocB toxin. However, differences between these N-domains do not result in purely exclusive behavior as the *E. coli* ClpX can support adaptor-dependent CtrA degradation and is able to restore growth defects in cells lacking SocB. In addition, an altered ability to process DnaX among the ClpX constructs suggest inherent differences in direct substrate recognition and may also reflect altered cooperation between the ClpX N-domain and AAA+ domain.

In conclusion, although the ClpX sequence is highly conserved between *E. coli* and *C. crescentus*, there are species-specific differences in activity that restrict the complementation between orthologs. These differences seem principally reflected by N-domain interactions, which account for both direct recognition and coordinated adaptor activity. However, it also seems that differences in substrate recognition by the ClpX AAA+ domain may affect how different ClpX orthologs support normal growth in *Caulobacter*. The work presented here argues that many aspects of ClpX function are conserved throughout bacterial evolution, but small differences in may result in an altered ClpX specificity that is only critical in a particular species.

## ClpXP proteolytic activity



**Figure 5.6 ClpX activities are defined by N-domain and AAA+ domain functions.**

(1) ClpX must interact with ClpP to enable proteolysis so that (2) Substrates directly targeted to or that have engaged the ClpX pore can be degraded. (3) Additional contact and selectivity by N-domain increases regulation through recognition and adapted delivery that can enhance stringency or change ClpXP substrate load. (4) Certain portions of the N-domain contain species-specific regions that target unique substrates. (5) Chimeric studies suggest that cooperation between substrate recognition by the N-domain and AAA+ domain have undergone optimization for species-specific activity.

## 5.5 Acknowledgements

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## 5.6 Materials and Methods

All *Caulobacter* strains, liquid or plated, were grown in PYE at 30°C, in the presence of the appropriate antibiotics or sugars.

### 5.6.1 *In vitro* ClpX analysis

ClpX and ClpP from *C. crescentus* and *E. coli* were purified as before [24]. Degradation of GFP-ssrA was performed as before [25].

### 5.6.2 *Caulobacter* strains

Expression of ClpX variants driven by the *Caulobacter* ClpX promoter were generated by cloning 500 bp upstream of the *Caulobacter clpX* gene and fusing this to ClpX alleles using a pMR10-based vector. Plasmids were electroporated into *ΔsocB*, *clpX::Ω* cells or parental strain UJ220 [16]. The following ClpX constructs were used: *Caulobacter* ClpX (CCX), *E. coli* ClpX (ECX), *Caulobacter* ClpX AAA+ domain (CCX minus the N-domain residues 2-53,  $\Delta$ N-CCX), and the chimeric fusion of the *Caulobacter* N-domain substituted for the N-domain on the *E. coli* ClpX body, a direct N-terminal 2-53 aa substitution (CC-ECX).

### 5.6.3 *Caulobacter* length analysis

Phase contrast images of *Caulobacter* cells (Zeiss AXIO ScopeA1) were subject to axial length analysis measuring pole-to-pole distance using the MicrobeJ software suite (ImageJ). Length is reported in microns.

### 5.6.4 ClpX depletion

ClpX depletion was done in a similar fashion to [20], except cells were back diluted twice during the ~20 hour ClpX depletion. Samples for ClpX replete conditions were taken prior to depletion. Samples for both ClpX replete and depletion conditions were pelleted and snap frozen

then re-suspended in an SDS loading buffer to a normalized OD<sub>600</sub> = 0.1. Sample volumes were then heated at 95°C for 5 min. Equal volumes of sample were subjected to SDS-PAGE followed by Western transfer. Resulting blots were probed with anti-ClpX or anti-DnaX antibodies and visualized with appropriate secondary antibodies conjugated to HRP and chemifluorescent substrate.

#### **5.6.5 CtrA degradation**

*ΔsocAB* and *ΔsocB, clpX::Ω* cells were diluted from overnight culture and allowed to reach mid-log phase, until the OD<sub>600</sub> reached 0.3-0.5 OD<sub>600</sub>. Translational inhibitor chloramphenicol was added to a final concentration of 30μg/ml. Following the addition of chloramphenicol, aliquots were removed every 30 min for 2 hours. Cells were pelleted and snap frozen then re-suspended to a normalized OD of 0.3. Sample volumes were heated at 95°C for 5 min. Equal volumes of sample were subjected to SDS-PAGE followed by Western transfer. Resulting blots were probed using an anti-CtrA antibody and visualized as above.

#### **5.6.6 Liquid growth assay**

*ΔsocAB* and *ΔsocB, clpX::Ω* with the corresponding plasmids were grown from single colonies. For the time courses, samples were back diluted to a starting density of OD<sub>600</sub> = ~0.1, and changes in optical density were measured over time. Resulting growth curves are the average of biological replicates, n=3. Error bars represent standard deviation for the set of n=3 (Figure 5.3D).

#### **5.6.7 Plated growth assays**

*ΔsocAB* and *ΔsocB, clpX::Ω* with appropriate plasmids were grown from single colony into log growth. All plating samples started with a density of ~0.1 OD<sub>600</sub> then followed a ten-

fold dilution for each subsequent spot. 4µl of resulting cultures was used to spot onto solid media and grown for ~3 days.

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**Table 5.1 Apparent binding constants between ClpX and ClpP using  $K_{\text{activation}}$  as a proxy.**

Values are derived from fitting degradation data for active proteases from Figure 1 to a first-order binding equation (degradation rate = maximum rate / ( $K_{\text{activation}}$  + [ClpP])). Because CCX + ECP does not degrade GFPssrA, we did not fit this data (N/D).

protease composition	$K_{\text{activation}}$ (nM)	maximum rate (/min/ClpXP)
<i>CCX + CCP</i>	9.1 +/- 1.1	0.57 +/- 0.02
<i>ECX + CCP</i>	12.1 +/- 3.3	0.92 +/- 0.08
<i>ECX + ECP</i>	24.1 +/- 5.5	1.06 +/- 0.09
<i>CCX + ECP</i>	N/D	N/D

## 5.8 Supplemental Information

**Table 5.2 Strains used in this study.**

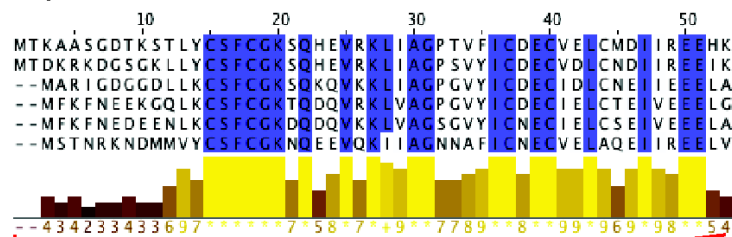
strain	description	reference
UJ200	CB15N <i>clpX</i> ::spec,xylX:ClpX(tet)	Jenal et al., EMBO 1998
CPC565	UJ200 <i>pkanR</i> (pMR10)	this study
CPC566	UJ200, pCCX	this study
CPC567	UJ200, pECX	this study
CPC568	UJ200, p $\Delta$ N-CCX	this study
CPC569	UJ200, pCC-ECX	this study
CAC219	NA1000 <i><math>\Delta</math>socAB</i>	C. Aakre/M. Laub (MIT)
CPC575	<i><math>\Delta</math>socB</i> , <i>clpX</i> ::spec	gift from P. Viollier
CPC570	<i><math>\Delta</math>socB</i> , <i>clpX</i> ::spec, <i>pkanR</i> (pMR10)	this study
CPC571	<i><math>\Delta</math>socB</i> , <i>clpX</i> ::spec, pCCX	this study
CPC572	<i><math>\Delta</math>socB</i> , <i>clpX</i> ::spec, pECX	this study
CPC573	<i><math>\Delta</math>socB</i> , <i>clpX</i> ::spec, p $\Delta$ N-CCX	this study
CPC574	<i><math>\Delta</math>socB</i> , <i>clpX</i> ::spec, pCC-ECX	this study

CLPX_CAUCR	1	MTKAASGDTKSTLYCSFCGKSQHEVRKLIAGPTVFCDECVELCMDIREEH	59
CLPX_ECOLI	1	MTDKRKDGSGKLLYCSFCGKSQHEVRKLIAGPSVYICDECVDLCNDIREEIK	60
		***. . : . ***** : ***** : * ***** *	
	60	KDGVPTPREICEVLDDYVIGQGHAKKVLAVAVHNHYKRLNHASKNNDVELAKSNILLVGP	119
	61	RSALPTPHEIRNHLDDYVIGQEAKKVLAVAVYNHYKRLRNGDTSNGVELGKSNILLIGP	120
		:. : *** : * : ***** : ***** : ***** . . . . * * * * . ***** : *	
	120	TGTGKTLLAQTLLARIIDVPFTMADATTLTEAGYVGEDVENIVLKLQAADYNVERAQRGI	179
	121	TGSGKTLLAETLARLLDVPFTMADATTLTEAGYVGEDVENIIQKLLQKCDYDVQKAQRGI	180
		*** : ***** : ***** : ***** : ***** : ***** . * * * * : * * * * *	
	180	VYIDEIDKISRKSDNPSITRDVSGEGVQQALLKIMEGTVASVPPQGRKHPQQEFLQVDT	239
	181	VYIDEIDKISRKSDNPSITRDVSGEGVQQALLKIEGTVAAVPPQGRKHPQQEFLQVDT	240
		***** : ***** : ***** : ***** : ***** : *****	
	240	TNIFICGGAFAGLEKIISARGA-AKSIGFGAKVTDPEERRTGEILRNVEPDDLQRFGL	297
	241	SKILFICGGAFAGLDKVISHRVETGSGIGFGATVKAKSDKASEGELLAQVEPEDLIKFG	300
		: : ***** : * * * * . . . ***** . : : * * * : * * * * : * * *	
	298	IPEFIGRLPVVATLEDLDEAALVKILTEPKNAFVKYQQLFEMENIGLTFTEALHQAQVAK	357
	301	IPEFIGRLPVVATLNELSEALIQILKEPKNALTKQYQALFNLEGVDLFRDEALDAIAK	360
		***** : * . * * * : * . ***** : ***** * * : * : * * : * * . : *	
	358	KAIARKTGAGRLRSIMEGILLETMFELPTYEGVEEVVNAEVEVEGRAQPLLIYAEKKGGA	417
	361	KAMARKTGAGRLRSIVEAALLDTMYDLPSMEDVEKVVIDESVIDGQSKPLLIYGKPEAQ	420
		*** : ***** : * . * * : * * : * * : * * : * * : * * : * * : * * : *	
	418	ASA-	420
	421	ASGE	424
		**.	

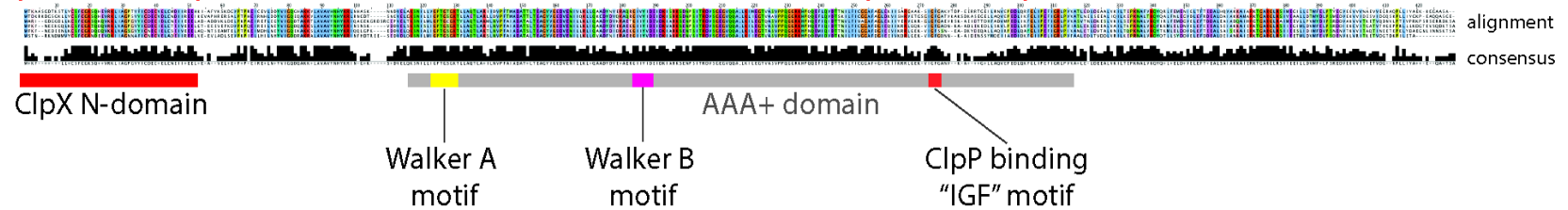
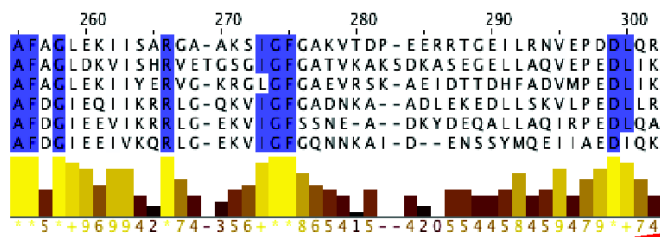
## Supplemental 5.1 Alignment of *Caulobacter* and *Escherichia* ClpX

The *E. coli* ClpX and *Caulobacter crescentus* ClpX share high homology (68% identity, identical residues marked by asterisks, similar residues with colons). The N-domain is shown in a dashed box.

## ClpX N-domain



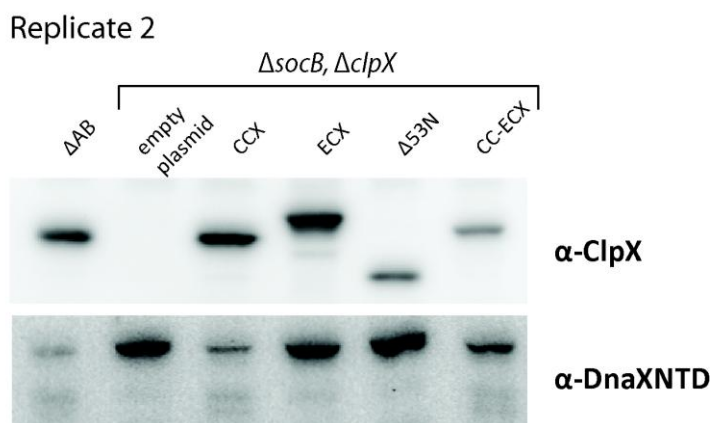
## IGF motif



## Supplemental 5.2 Protein alignment of select bacterial ClpX

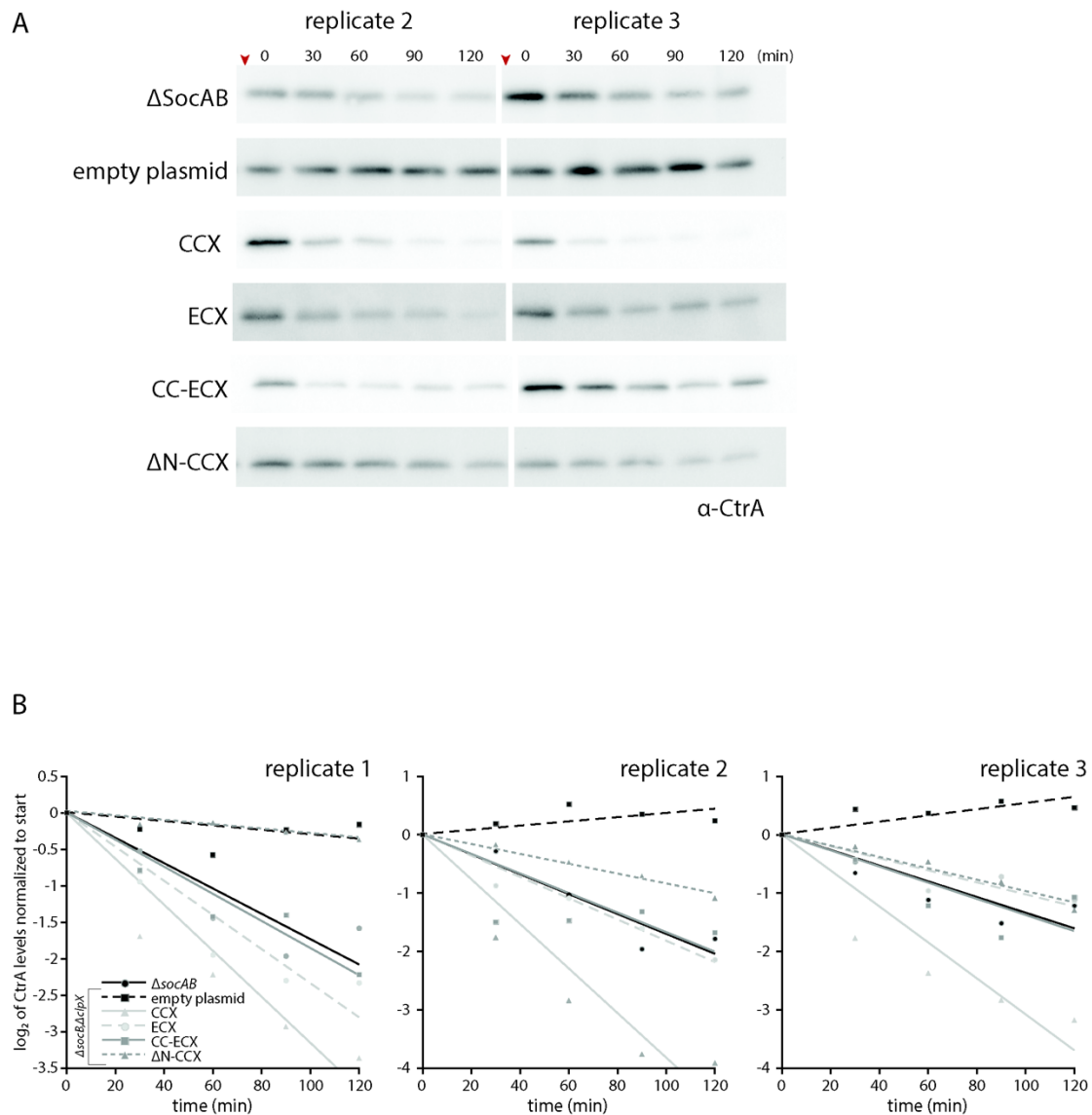
An alignment of ClpX proteins (*C. crescentus*, *E. coli*, *M. tuberculosis*, *B. subtilis*, *S. aureus* and *S. pneumoniae*) shows high conservation for amino acid residues constituting the N-domain. Blue shading highlights identical residues. The AAA+ domain also bears high sequence conservation, while the region surrounding the conserved IGF motif required for ClpP interaction is less similar. Yellow columns (labeled consensus) in inset denote conservation with larger numbers denoting higher conservation. Black columns in the full sequence (labeled consensus) denote residue similarity across the protein.





**Supplemental 5.4 Only ClpX constructs with the *Caulobacter* N-domain correctly process DnaX**

In support of Figure 5.4. Replicate of anti-DnaX westerns of  $\Delta socB, \Delta clpX$  cells with ClpX variants expressed as labeled. Similar to the blot shown in Figure 4, the steady state levels of full-length DnaX during logarithmic growth is higher in cells that lack the CCX N-domain compared to wildtype and cells expressing ECX alone fail to generate the  $\gamma 2$  form of DnaX.



### Supplemental 5.5 replicates for CtrA half-life per ClpX variant used

In support of Figure 5.5. (A) Replicates of the translational shutoff experiments. (B). CtrA levels were quantified relative to ClpP levels and plotted as log<sub>2</sub> levels normalized to time zero. X-axis is time after antibiotic addition. The three independent slopes from the plots of each time course were averaged to determine the half-life and standard deviation for the table reported in Figure 5.5A.

## 6. OUTLOOK FROM KNOWN DNAX INTERACTIONS TO WHAT SHAPES THEM

### General Chapter Overview

Often, when orthologous proteins retain similar shape, they adopt similar activity or function. Clamp loaders exemplify this structure-function relationship seen by its conserved activity across organisms. Conserving activity exists for both sliding clamps and the loaders required for their placement and removal on DNA despite sequence and oligomerization differences [1,2].

Activities and interactions establish the conserved function of protein DnaX in *E. coli*. DnaX composes three-fifths of the pentameric core clamp loader that contains several key attributes that are required for replication activity while providing core contacts that ensures the loader function as a synchronized unit. The loader portion provides contacts that powers DnaX based loading, while interactions in passive, tethering C-terminal domain increases the number of clamp placement sites. Directing the clamp loading through the C-terminal domain effectively targets clamps to precisely where they are needed. However, loader activity may change based on cellular cues and environmental effectors that require diverse clamp interactivity. Some loader activities need to remain constant, regardless of the cell's request for changes in clamp demand. The loader's ability to discern between required activity while remaining flexible for reacting according to the cell's needs may be directly gated through its non-conserved interactions.

Regulating clamp activity varies organism to organism, and correlates to the loader and loading network supplied by that organism. Here, changes in clamp activity originates from protein-partner interaction that are influenced by modifications to clamp, loader, or supplied effector proteins. Eukaryotes employ a complex system that provides both a host of posttranslational chemical modifications to clamp and loader, and separate loader subunits for



specific responses that direct clamp loading efforts. Prokaryotes do not have the luxury of extensive posttranslational modification or range of subunits that form a layered eukaryotic response. Instead, prokaryotic model *E. coli* uses a tethering C-terminal domain that facilitates all required contacts for facilitating clamp loading, yet prokaryote *Caulobacter* lacks conservation in its poorly conserved, C-terminal domain IV. The persistent appearance of a third version of loader gamma in *Caulobacter* suggests a meaningful production, where this additional form provides alternate properties that help satisfy a need in loader activity.

## **6.1 Replicative role of DnaX, a background**

The clamp loader mainly functions to correctly place clamp usage and activity. Clamps themselves comprise a homodimer (prokaryotes) or homotrimer (eukaryotes and some archaea), and but retain conserved structure that requires loader interaction with the major hydrophobic C-terminal groove of the clamp for placement and removal [3,4]. Loader composition remains conserved in subunit number, overall shape, and conformational changes based upon ATP binding or hydrolysis (reviewed in [5]). The types of partner proteins and activities mediated by sliding clamp reflect its ubiquitous function across species, just as the composition and activity contained by loader complex does. The loader's ability to open clamp, recognize correct placement cues, and rearrange the local nucleic acid architecture are conserved activities required for effective clamp use [1]. Recognition of primer/template junctions occurs most frequently during lagging-strand synthesis where the loader interacts with repeat junctions, and delivers clamp at every primed ~1200bp produced during replication [6].

The *E. coli* clamp loader provides the clamps required for lagging-strand synthesis through extra C-terminal contacts confined to the essential tau portion of the DnaX protein [7] that tethers the clamp loader to both helicase and polymerase [8]. Tethering the loader to fork machinery ensures fast clamp loading at each primer-template junction. Additionally, these C-

terminal contacts couple leading and lagging strand polymerases to the replication fork [9], affecting their processivity [10]. Binding of the DnaX C-terminal region to helicase also increases helicase speed [11], suggesting the loader enhances helicase activity, and therefore loader tethering indirectly controls the speed of replication. In context, lagging strand growth and the clamp loading required for its completion must correctly correlate to how the replisome progresses, and is ultimately determined by the synthesis speed of the leading strand. Replicative polymerases synthesize new leading strand DNA at a rate correlating to the dwell time of clamp bound to the tethered loader (~1sec, [12]), and the time between successive Okazaki fragments (~1200bp [6]). The loader coordinates replication through the loading and tethering domains, controlling the speed of the leading strand and providing clamps for lagging strand synthesis. For the lagging strand, ATP hydrolysis stimulated by the loader's recognition of primer-template junctions ([8]) accurately fine-tunes clamp release at this interface. After clamp release, the loader must prepare for a new clamp to be placed at the next primed event. This triggered clamp release coordinated by DnaX at recognized primed junctions ensures clamp availability at every Okazaki fragment generated.

The requirement for unloading rivals the loading process, as the loader must also remove clamps placed onto DNA in a timely fashion. The lagging strand produced by the semi-discontinuous nature of replication serves as a clamp sink, decreasing the number of available clamps as replication continues. For example, the number of clamps found in *E. coli* (approx. 200-300 clamps [13]) cannot cover the ~3900 primed Okazaki fragments formed during replication. These numbers imply each clamp must be recycled a minimum of ~13 times just to cover the quantity of primer-template junctions produced, assuming that leading strand synthesis remains uninterrupted. Since clamps spontaneously fall off DNA extremely slowly [14], clamp recycling requires the unloading properties of the clamp loader.

## **6.2 Loader activity during DNA damage response, changing clamp interactions**

Imperfections in synthesis constantly challenge DNA integrity. Repair to mismatches, gaps, breaks, and environmentally caused DNA insults each require a different subset of proteins designated for their restoration. Repair proteins require clamps during these corrective changes to localize and increase their efficiency. Clamps provide a platform at the damage site, effectively tethering and enhancing repair activity. Eukaryotes and prokaryotes differ in their approach by ensuring the correct partitioning of clamp activity between replication and repair. Eukaryotic loaders lack the extra C-terminal extension found in the prokaryotic loader. Instead, eukaryotes rely on dedicated interchangeable subunits of the loader (called replication factor C, RFC) tasked for this purpose. Additionally, these subunits can undergo reversible modifications (phosphorylation, ubiquitination and SUMOylation, etc.) that influence partner protein interactions and change loader activity. These decorations adorn both the clamp (PCNA [13,15,16], reviewed in [17]) and the loading complex (RFC, reviewed in [18]), effectively changing loader-clamp interaction in response to DNA stress [19] and coordinating loader activity with replication [15]. The RFC core loader forms a heteropentamer of five separate subunits (RFC1-5) that change activity depending on the occupancy and modification state of the large subunit position held by RFC1 [18,20]. For example, substitution of RFC1 with Rad24 as the Rad24-RFC loader is not used during replication but has selectively loads the non-canonical hetero-trimeric clamp called 9-1-1 that is required for loading clamps at damage locations such as double-strand breaks. The Rad24-RFC loader also functions as a PCNA unloader, suggesting the activity this unloader has triggered during DNA damage actively prevents or stalls ongoing replication ([20], pages 923-929).

## **6.3 Less regulation, same activity**

Using multiple versions of Replication Factor-C along with using posttranslational modifications creates a flexible response network that handle the simultaneous cell demands in

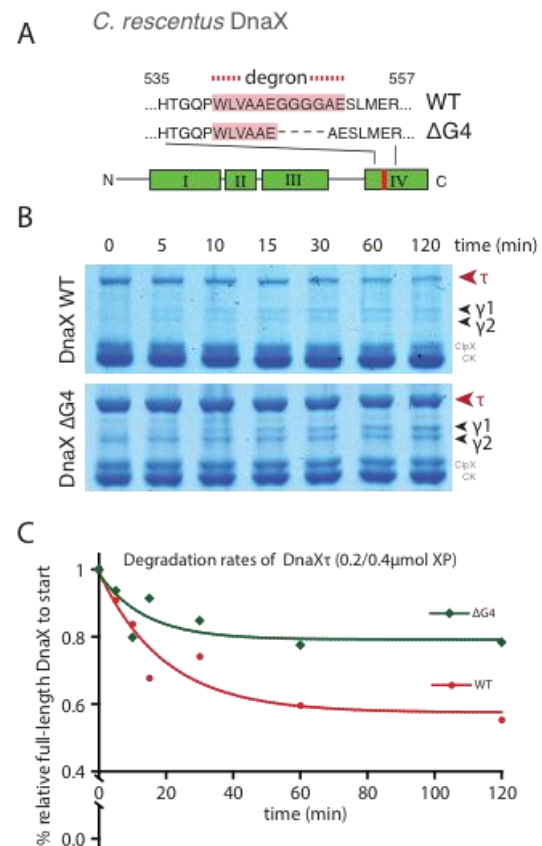
eukaryotes that require clamp and loader activity. Prokaryotes like *E. coli* lack these extra regulatory modifications and loaders. Consequently, only one prokaryotic loader exists to facilitate total loading activity, and must supply the clamps required for both replication and repair. Here, a combination of loader interactions drive clamp placement; such as primer-template junctions, occupancy of clamp's hydrophobic cleft [8], and other proteins that recruit the loader for clamp placement [21,22]. Replication and repair proteins found in *E. coli* lack the extravagance of extensive modifications, such as ubiquitination and SUMOylation, used to switch activity. Instead, a more modest set of posttranslational modifications influence activity, through use of effector proteins or changes in protein concentrations.

*Caulobacter crescentus* and *E. coli* share many parallel cellular processes, proteins, protein activity, and interactions. However, several attributes make *Caulobacter* a wonderful system to understand cellular processes not undertaken by *E. coli*. For example, *Caulobacter*'s cell cycle program requires tight control of replication to time a single round per cell division, and coordination of a morphological differentiation that coincides with replication competency. *Caulobacter* utilizes targeted proteolysis to accomplish these tasks, such as initiating the transition to replication competency, and triggering the start of cell cycle differentiation (reviewed in [23]). The expression of the proteins required for genome duplication just prior to initiation ensures enzyme availability for successful replication [24,25]. The timing of *Caulobacter*'s cell-cycle differentiation with replication may also require specific regulation for loader activity and clamp placement that mirrors a level of coordination similar to eukaryotic loader activity.

*Caulobacter* uniquely employs proteolysis to generate an alternate form of the clamp loading protein, DnaX. Partial proteolysis generates two smaller DnaX isoforms in *Caulobacter* that are needed for loader function during DNA damage [26], preserving similar diversity generated by translational slippage that generates a smaller DnaX form in *E. coli* [27] that also enhances the DNA damage response [26,28-31]. Degradation joins a number of posttranslational modifications that play an important role during the oxidative stress response. Proteolytic events respond to external cues triggered when environmental conditions challenge normal growth. For example, cells entering the start of the SOS response begin as a triggered event started by RecA auto-cleavage, which continues to propagate in response to DNA damage (reviewed in [32]).

#### 6.4 How does proteolysis change protein activity and what are the minimal limitations for this change?

As a posttranslational modification, degradation changes direct interactions permanently by limiting existing or uncovering new, previously shielded contacts. Regardless, since proteolysis is an irreversible response, the ensuing degradation must be warranted. In this case, partial proteolysis exposes otherwise



**Figure 6.4 Adjacent residues C-terminal to the required di-alanine pair affects DnaX degradation**

A. Removal of the following glycine residues in the downstream portion of the degon (B) significantly reduces degradation of DnaX $\tau$ , which degrades less completely than DnaX-WT. C. Quantification of DnaX $\tau$  degraded in (B).

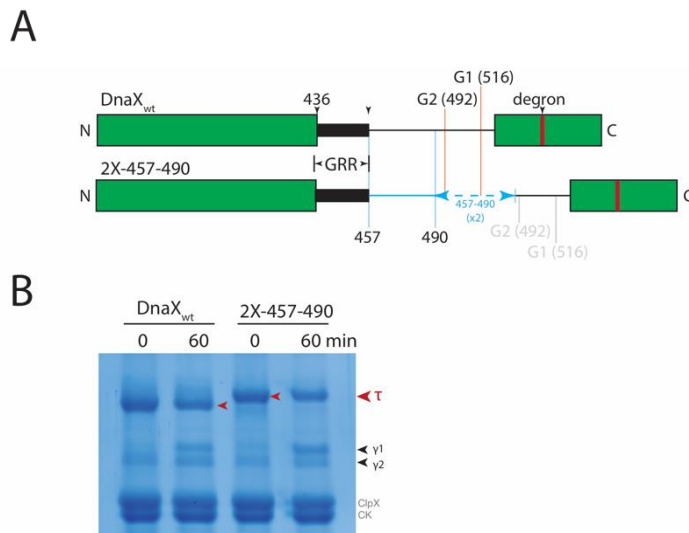
masked binding interfaces or limits interactions to remaining available protein-protein contacts. Protein binding consists of the compatible pieces that effectively tether the proteins together. For example, the C-terminal domain of *E. coli* DnaX directs Pol III assembly where productive binding organizes replication fork polymerases [8]. Remaining contacts contained within the C-terminus also accelerate helicase speed *and* connects active clamp loading to the growing fork, ensuring clamp availability tracks as DNA synthesis progresses [9,11]. Similar to the tau-specific tethering interactions that bind the *E. coli* loader to the replication fork, the N-terminus of translesion bypass polymerase UmuD binds sliding clamp's hydrophobic groove, effectively tethering UmuD to DNA. During induction of the SOS pathway, RecA auto-cleavage combined with the presence of ssDNA stimulates UmuD auto-cleavage, and creates UmuD'. This self-cleavage trims the N-terminal ~24aa, and removes the UmuD clamp-binding sequence [33]. Essentially, this length of peptide contains both the minimal requirement for clamp contact and an interactive cleavage site.

Perhaps we can extrapolate using UmuD as a model for a removable binding tag. The ~24aa stretch contains a clamp binding and an additional cleavage sequence within this peptide length. A priori, if ~24 aa satisfies the constraints for binding clamp and contains an additional cleavage sequence, the size difference between *Caulobacter*'s two gammas may also contain unique contacts. UmuD cleavage may model this proteolytic gated interaction that changes its clamp binding to non-binding upon cleavage, where changes in binding rests within trimming of as little as ~24aa. However, it is possible that either the peptide sequence or requirements for the positioning of a cleavage event may differ between terminally located cleavage modules compared to those located between domains or within inter-domain linkages. Using *Caulobacter* DnaX as a model for internal or inter-domain recognition reveals that changing the adjacent sequence required for recognition plays an important role in the overall degradation of the protein (Figure 6.4A). Removing 4 glycine residues (DnaX<sub>ΔG4</sub>) directly downstream of the recognition

sequence changes degradation characteristics (Figure 6.4B,C). I speculate that this deletion mutation limits degron flexibility or recognition, which ultimately reduces accessibility and degradation by the ClpX machine compared to wildtype protein both *in vitro* and *in vivo*. The reduction in resulting substrate degradation suggests that perturbations within the degron negatively impact recognition, when recognition drives degradation.

## 6.5 Does size matter? Asking if specificity exists between *Caulobacter* gammas

The peptide size difference between *Caulobacter*'s two DnaX gammas may be stochastically generated by ClpXP partial proteolysis. However, generation of two gamma isoforms creates one



**Figure 6.5 Increasing the linker between the GRR and the degron does not affect processing**

A. Cartoon representing the increase in linker length addition. Also provided is the potential expected relocation of a new  $\gamma 1$  and  $\gamma 2$  positioning. B. *In vitro* degradation of the 2 X-457-490 proteins shows production of the same two gamma fragments formed in wildtype processing.

gamma with extra C-terminal coverage extending into the tau-specific portion of DnaX, that *just happens* to be advantageous for dealing with DNA damage [26]. A better question is asking ‘what extra contacts do these extra ~24 amino acids afford?’ Basic local alignment of *Caulobacter* DnaX

with *E. coli* DnaX reveals this extra ~24aa residue distance that remain

on the larger generated gamma isoform covers the same portion known in *E. coli* DnaX to

exclusively bind helicase. As a caveat, this speculation fails to

account for the lack of *Caulobacter* DnaX sequence conservation compared to the *E. coli* C $\tau$ ,

where *Caulobacter*'s C $\tau$  lacks the natural partition into separate C-terminal domains IV and V as gammaproteobacteria do. This poor C-terminal sequence conservation masks the exact location of helicase and polymerase binding, which suggests these interactions may not exist within the *Caulobacter* DnaX C-terminal domain IV. This data is currently unknown; however, domains I-III alone are insufficient for *Caulobacter* viability. Formally testing the boundaries and binding requirements between the C-terminal region and expected partners would explicitly determine whether the peptide section separating these two gammas facilitates a selective interaction. Here, validation could be performed by a crosslink or pull-down approach using the inter-gamma distance as bait. Validation of prey proteins can be done using MS-MS, or by Western blotting against tagged helicase/polymerase proteins expressed *in trans*.

Why are two gammas generated in *Caulobacter*? The possibility exists that the defined length by which *Caulobacter* gamma differs is directly produced as originates from the initial point of recognition within the C $\tau$ . *In vitro* data shows that doubling the linker length of DnaX between the glycine rich region (GRR) and recognition element still results in two isoforms (Figure 6.5A). The size difference of isoforms generated between the wildtype and 2Xmutant persists after ClpXP degradation (Figure 6.5B). Both DnaX proteins generate the same size isoforms and are insensitive to changing size upon additional linker length. This data suggests that elements N-terminal to the degron (the GRR or tightly folded loading domain) determine the production of two gammas.

It is possible that both gamma forms are generated simultaneously, where the size difference is a stoichiometric product of ClpXP engagement and subsequent processing. The exact process that yields two smaller isoforms of DnaX is unclear. I speculate that the organization or oligomerization of the DnaX C $\tau$  and the required delivery by the ClpX N-domain influences generating two DnaX fragments of defined length. SEC analysis of the DnaX C $\tau$  reveals that the DnaX  $\tau$ -specific domain dimerizes, and by doing so may present *more than one*



recognition sequences to the N-domain of ClpX. Having two degrons present may 1) orient C $\tau$  translocation creating an initial ClpX recognition preference that propagates into the ClpP peptidase chamber or 2) establish a requirement for multiple contacts by the ClpX N-domain to coordinate self-adapted delivery (Figure 6.6). In scenario 2, perhaps the DnaX<sub>ΔG4</sub> mutant lacks enough peptide sequence from each degron to span multiple ClpX N-domain contacts required for efficient, self-adapted delivery, therefore inhibiting degradation (Figure 6.4).

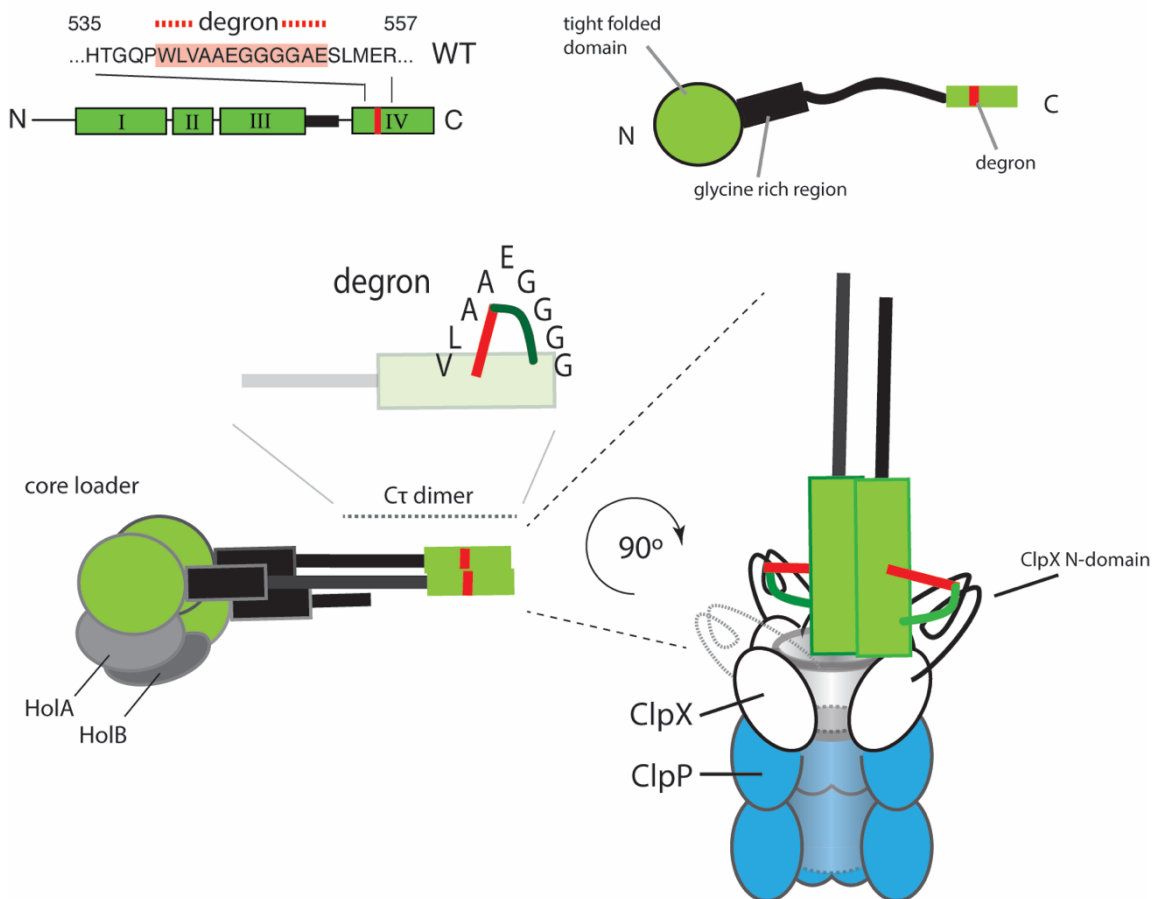
## 6.6 Why are two gammas generated in *Caulobacter*?

The possibility exists that the defined length separating *Caulobacter*'s two gamma proteins directly originates from C $\tau$  recognition. *In vitro* data show that doubling the linker length of DnaX between the glycine rich region (GRR, Figure 6.5) and recognition element still produces two isoforms equal in size to that of wildtype (Figure 6.5B), suggesting ClpXP processing is insensitive to the additional linker length. This data suggests that elements N-terminal to the degron (the GRR or tightly folded loading domain) determine the production of two gammas.

Consider if both gamma forms are generated simultaneously, and perhaps the size difference is a byproduct or limitation of ClpXP processing. Whether the organization or orientation of the DnaX C $\tau$  is independent of or if the ClpX N-domain dependent delivery generates the two gamma isoforms in *Caulobacter* remains unclear. SEC analysis of the C $\tau$  reveals an ability for the DnaX  $\tau$ -specific domain to dimerize, therefore presenting *more than one* recognition sequences to the N-domain of ClpX. Having two degrons present may 1) orient C $\tau$  translocation creating an initial ClpX recognition preference that propagates into the ClpP peptidase chamber or 2) establish a requirement for multiple ClpX N-domain contacts to coordinate degradation through self-adapted delivery (Figure 6.6). In scenario 2, perhaps the DnaX<sub>ΔG4</sub> mutant lacks enough peptide sequence from each degron to span multiple ClpX N-

domain contacts required for efficient, self-adapted delivery, therefore inhibiting degradation (Figure 6.4).

### *C. rescentus* DnaX



**Figure 6.6 A schematic representation of DnaX C-terminal delivery to ClpXP**

Here, dimerization of the DnaX C-terminus provides multiple contacts for two ClpX N-domains to engage. Doing so facilitates both efficient delivery to the protease and orients the DnaX C-terminus for degradation.

## 6.7 Bacterial loaders are Swiss-Army knives for clamp placement

*Caulobacter* produces tau and both gammas throughout all parts of cell growth regardless of damage, replication or developmental state. The constant presence of all three forms suggests the *Caulobacter* clamp loader always exists and satisfies all loading and clamp related activity as a composite complex. Similarly, *E. coli* studies show that the stochastic distribution of holoenzyme complexes that contain gamma always contains tau. This data suggests that loaders composed solely of gamma, although shown to complete many functions *in vitro*, are most likely not present *in vivo* [31].

Why produce the gamma protein then? Previous work demonstrated that effective chromosome partitioning in *E. coli* requires  $\gamma$ , seen in the use of temperature sensitive *parE10* allele mutants. Successful recovery of these *parE10* allele phenotypes show that over expression of  $\gamma$  is able to resolve genome concatenations in these mutants cells under non-permissive conditions, essentially substituting the activity normally reserved for Topo IV [34]. Taken together, the absence of a  $\gamma$ -only loader suggests that hetero-oligomers of DnaX in the clamp loader performs all required genome maintenance, organization, and activity, in spite of constant presence of maintaining its C-terminal domain contacts. My data supports the existence of similar mechanisms in *Caulobacter*, an organism that undergoes constant genome maintenance and accommodates a tightly controlled replication initiation. *Caulobacter* utilizes all three DnaX forms swimming along in its sunlit, DNA damage inducing, freshwater home. Perhaps demands of clamp loading and placement require that all the necessary tools perpetually be ready in advance. Considering the absence of modifiers known to change loading activity (ubiquitin and SUMOylation), bacteria may rely more heavily on having loader activity regulated by partial proteolysis or translational frame-shifting. The clamp loading portion of the loader complex retains these conserved loader functions, and must remain constant as only the loader is tasked with covering specific activities, such as recognizing primer-template junctions, and opening

clamp for placement and removal. Aside from loader's involvement with clamp interactivity, the DnaX C $\tau$  functions to passively tether the loading complex while enhancing the activity of the proteins it tethers. Combining these functions consolidates the need for regulation by increasing the universality of the bacterial loader. Both *Escherichia* and *Caulobacter* can exist with tau-only versions of DnaX ([35], Appendix Figure 7.3B), suggesting tau-alone sufficiently supports normal growth. The importance of gamma emerges during when DNA is challenged, and when the need for maintenance or repair arises.

## 6.8 References

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## APPENDIX

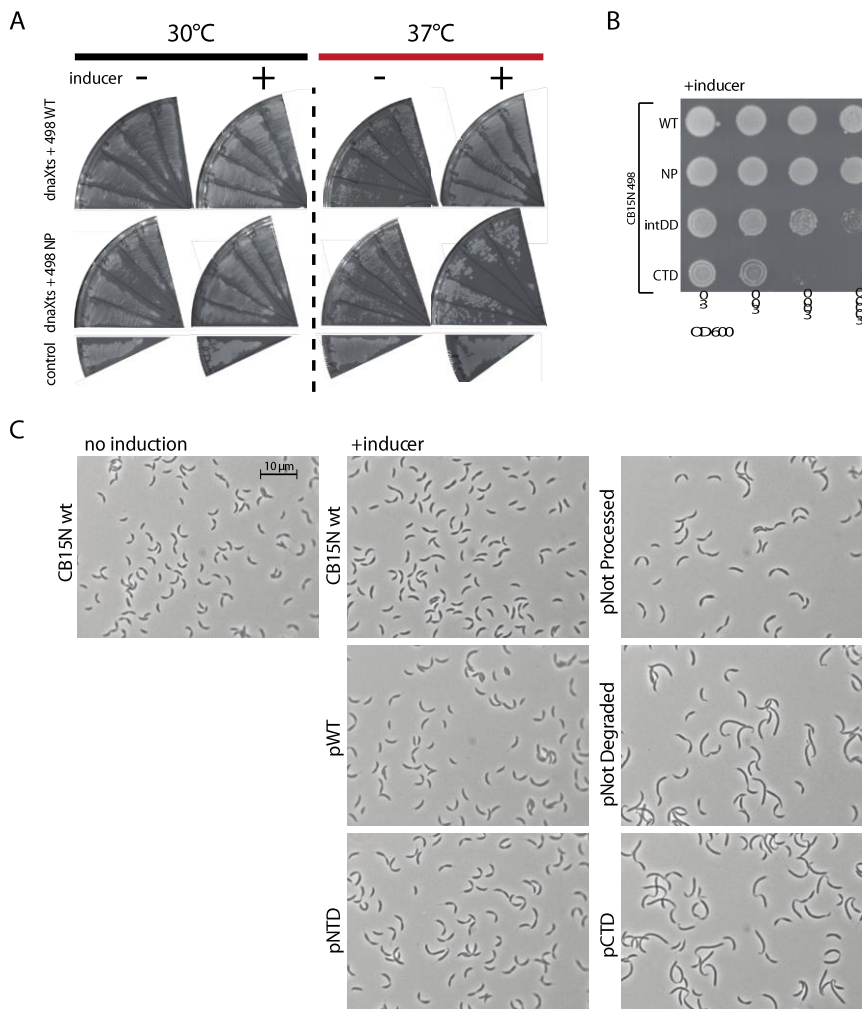
### A. ADDITIONAL DnaX/ClpX CHARACTERIZATIONS

#### A.1 Why does full-length DnaX fail to complement the DnaX<sub>ts</sub> phenotype?

The first portion of the appendix takes a more in depth look at DnaX in *Caulobacter crescentus*, and characterizes it in a more targeted fashion. Here, I attempt to take a more in depth look at DnaX for circumstances that govern partial proteolysis and consider factors that influence the ClpXP generation of alternate DnaX forms. Why are two gamma isoforms generated, and are there any specifics that can be assigned to this process? *Caulobacter* requires both the full-length and processed forms of DnaX, and suggests that providing multiple DnaX forms is beneficial for *Caulobacter* survival. (Figure A.1A, in a DnaX<sub>ts</sub> background) Only when DnaX<sub>wt</sub> is produced *in trans* and is processed down into gamma do cells exhibit robust growth. This data suggests *Caulobacter* survival depends on having multiple DnaX forms. By utilizing a mutation that makes a temperature sensitive DnaX found within the ATPase domain is located at <sup>G118<sup>D</sup></sup> (corresponding to a well characterized, *E. coli* mutation existing at the same locale [1]) non-permissive temperatures inhibits cell growth.

In a corollary experiment, wildtype cells that overexpress from plasmid either a non-degraded (intDD) mutant version or the highly degraded DnaX C-terminus alone (residues 457-608) are sensitive to overexpression, and pay a growth penalty (Figure A.1B). Similarly, cells that express either the DnaX C-terminus or a non-degraded version of the protein have reduced plated growth and have an expression phenotype seen as cellular elongation (liquid growth, Figure A.1C). These data suggest that not only recognition but also the action of ClpXP trimming of the DnaX C-terminus positively influences cell survival. I speculate that the reduction in growth seen in the non-degraded (intDD) mutant may be reflected by artificially stabilizing the C-terminus in

spite of the fact that the C-terminus (residues 457-608) degrades rapidly. Trans expression produces the M2-C-terminus, which may prevent proper regulation of endogenous DnaX by ClpXP, effectively altering proper tethering and clamp-loader interactions, such as binding polymerase and helicase.



**Figure A.1** Proteolysis of the DnaX C-terminal domain is required for normal growth

A. *Caulobacter* cells that express wildtype DnaX (DnaX<sub>WT</sub>) in trans can complement a temperature sensitive mutation in endogenous DnaX at non-permissive temperatures. A mutant version of DnaX (DnaX<sub>NP</sub>) that is not processed into gamma cannot complement this phenotype. B. Overexpression of DnaX mutants in trans differentially affect cell growth. Plasmid expression of wildtype (WT), a non-processed form (NP), a protease insensitive mutant (intDD), and just the tau-specific portion (CTD) were expressed in a wildtype background. Both the stable intDD and expression of the CTD reduce plated growth, suggesting too much of the DnaX C-terminal domain negatively affects *Caulobacter* growth. C. Negative growth observed with the increased presence of the DnaX C-terminal domain exhibits an elongation phenotype not seen in DnaX mutants that can be recognized by the ClpXP protease.

## **A.2 DnaX exists stably as three forms all the time; DnaX levels negatively feedback into the system.**

Previously, we have shown that DnaX exists in three forms, a full-length and two smaller, N-terminal gamma fragments ([2], from supplemental Figure 2A). Induction of M2-DnaX is seen as three components (Figure A.2A top) that accumulate over time (Figure A.2A bottom). DnaX exists as three forms throughout the duration of *Caulobacter*'s cell-cycle lifetime, and remains consistent as it progresses from the motile form to the stalked cell type (Figure A.2B). This data suggests that all three DnaX forms are present irrespective of *Caulobacter*'s progression through its life cycle, exist during this transformation, and meets the changes in DNA metabolic requirements through the transition from being replication incompetent to becoming replication competent.

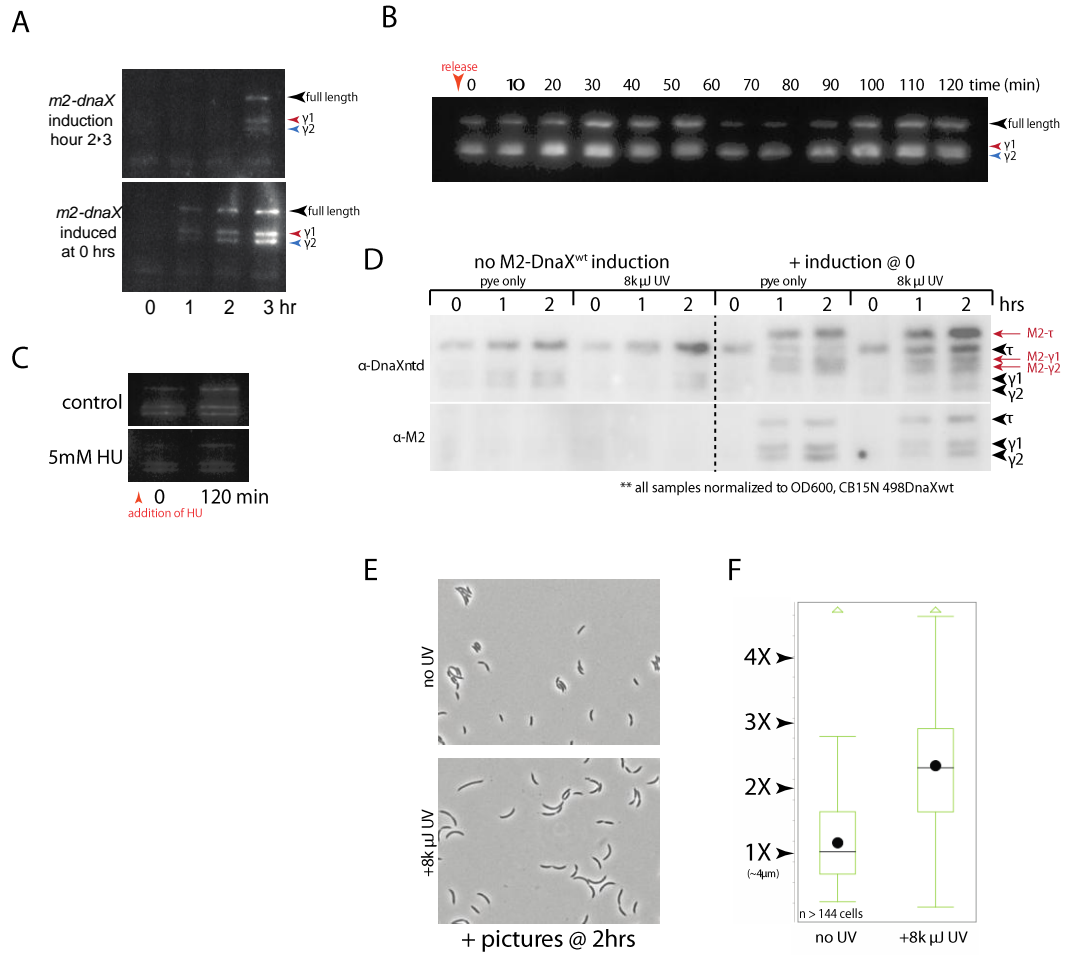
Just as multiple DnaX proteins are present throughout *Caulobacter*'s life cycle, all three forms are represented when DNA is challenged with repair. No appreciable change in response seen by changes in protein levels or DnaX processing occurs, suggesting clamp-loading system remains consistent even when various forms of chemical (hydroxyurea, MMC, MMS, Figure A.2C) or UV damage (Figure A.2D) are applied. Here, the resulting processing and distribution of tau (DnaX full-length) to gamma (ClpXP processed DnaX) slightly shifts but does not show any isoform preference. This slight shift is not surprising as the response to DNA damage causes the >2 fold transcriptional upregulation of *dnaX* [3]. The extra protein produced in response to damage necessary for resolution may be partially limiting the ClpXP capacity for degrading or processing DnaX, and directly manifesting when ClpXP is required to handle increased specificity during periods of higher substrate load. For another example, cells pulsed with UV damage show a characteristic DNA damage phenotype consisting of an elongated morphology, and increased induction of RecA levels (elongation example in Figure A.2E, quantification in A.2F). Taken together, DnaX processing continues under both normal conditions and under DNA

damage conditions, with a relative proportionality of DnaX levels in all three forms remaining constant. These data are consistent with all three DnaX forms being capable of maintaining activity required for normal DNA metabolism, where the extent of DnaX processing remains persistent during maintenance, repair, and through *Caulobacter* cell differentiation.

### **A.3 Removing ClpX allows *Caulobacter* to exist with DnaX<sub>tau</sub> alone, *Caulobacter* can support a tau-only construct.**

Our work has shown that the full-length, tau form of DnaX is not the sole species in *Caulobacter*, but is always accompanied by the presence of two smaller gamma forms. Work from the Laub group at MIT identified that the essential nature of ClpXP in *Caulobacter* resides in its ability to degrade the clamp-binding toxin, SocB [4]. Removal of this toxin allows for the deletion of ClpX, which also abolishes DnaX processing; producing cells that die easily and grow poorly. Subsequent loss of gamma happens upon depletion of ClpX or ClpP so that only the full-length form is seen in cells, suggesting that even when sick and devoid of the ClpXP protease, cells can survive with the Tau gene product alone only when toxin SocB is absent (Figure A.3A). One speculation is that in this  $\Delta$ ClpX,  $\Delta$ SocB background, since DnaX is neither processed nor degraded, its full-length form accumulates in cellular concentration. If additional full-length DnaX is supplied *in trans*, the extra protein suppresses cell growth ([5], Figure 1). This suggests transcriptional regulation maintains DnaX at the level required for survival below the maximal DnaX concentration tolerated by the system. However, it is unknown whether *Caulobacter* survives with solely the full-length form of DnaX under normal or wildtype conditions.

The ability for *Caulobacter* to grow with only DnaX full-length was tested using a gene-displacement technique that incorporated a non-degraded mutant (DnaX<sub>intDD</sub>) at the endogenous *dnaX* locus. The DnaX<sub>intDD</sub> variant mimicked previous conditions that support cell survival in the  $\Delta$ ClpX,  $\Delta$ SocB strain, but explicitly tested survival *in a wildtype background*. This strain also provides a platform for exploring the biological relevance of generating gamma in *Caulobacter*,



**Figure A.2 All three DnaX forms are stable under DNA damaging conditions, concentrations of the full-length protein provide feedback for maintaining protein levels**

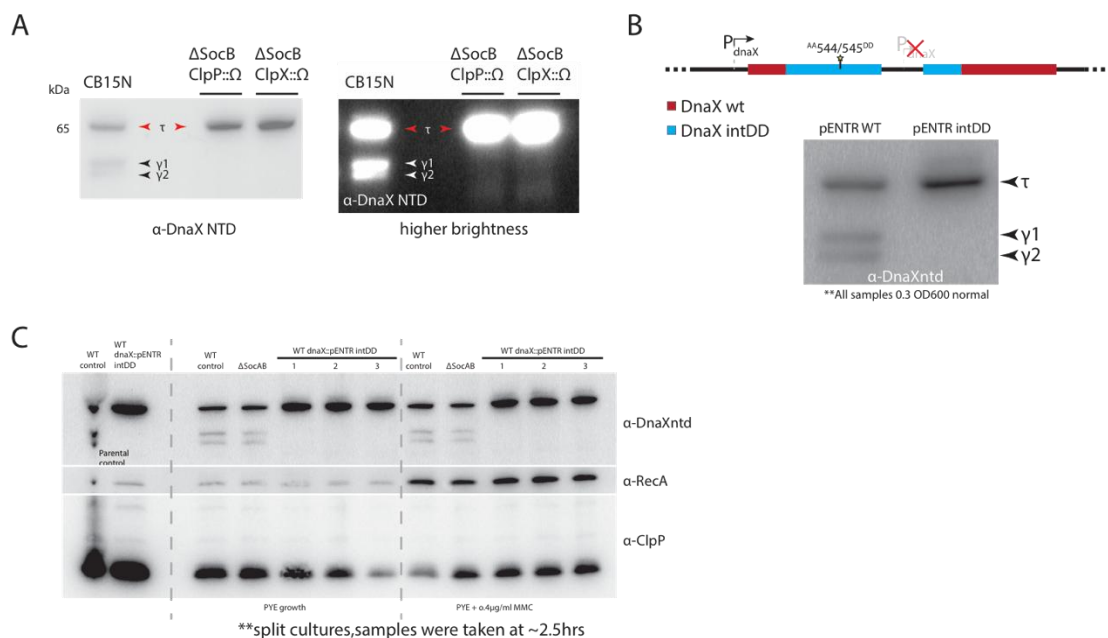
A. Whether newly translated DnaX is introduced into the system or accumulates over time, DnaX expression exists in three forms. B. Synchronized *Caulobacter* cells maintain consistent DnaX levels and representation of tau with two shorter gammas. C. Addition of hydroxyurea in concentrations that deplete dNTP pools and induce the DNA damage response system does not change DnaX processing. D. DnaX levels increase as a response to UV damage. Co-expression of M2-tagged DnaX during normal growth and under UV damage shows active processing still occurs after damage and during repair. Interestingly, constant M2-DnaX induction suppresses endogenous tau production over time. This suppression suggests *Caulobacter* regulates DnaX production to maintain healthy operational levels. E. Cells exposed to UV have an elongation phenotype, and more than double in length on average (quantified in F). Both E and F correspond to hour 2 of induced cells in (D). Over expression of M2-DnaX does not cause an elongation phenotype in *Caulobacter*.

and allows for directly testing the importance of DnaX processing (Figure A.3B). If *Caulobacter* tolerates the full-length form as the only DnaX protein available, loss of gamma would be exhibited in two potential categories that affect *Caulobacter* growth; 1) changes during normal growth and maintenance or 2) changes during DNA stress, damage, and repair. Generating this strain would answer directly whether having gamma benefits normal, everyday clamp-loading operation, or if a subset of clamp based-activity that responds under special conditions requires gamma.

I was able to successfully integrate the tau-only mutation at the endogenous *Caulobacter dnaX* locus; where integration did not prevent cell growth, change cell cycle differentiation, or morphology. This mutant strain behaves similarly to normal, wildtype *Caulobacter* cells. Furthermore, the mutant strain responds to DNA damage, exhibits similar morphology, and responds with RecA induction levels to comparable damage in wildtype cells. These data suggest that the tau-only DnaX form can be stably expressed in the wildtype background from the *dnaX* locus, and remains stable under DNA damaging conditions, without eliciting or suppressing the RecA SOS response (Figure A.3C).

#### **A.4 Gamma expression in trans suppresses same form production and is not tolerated in a tau-only background.**

My initial assumption was that some regulatory change must accommodate the ability for *Caulobacter* to tolerate the tau-only mutation. Genomic compensatory mutation(s) would most likely allow for survival with only expression of the full-length DnaX mutant allele. To test this hypothesis, I analyzed the tau-only mutant using Next Generation Sequencing (NGS), which suggested ~7 mutation sites in the mutant background. Interestingly, none of these mutation sites directly relate to DNA metabolism, replication or repair (Table A.1). This data suggests that *Caulobacter* actively adjusts to having only full-length DnaX without drastically changing the proteins required for maintaining DNA metabolism.



**Figure A.3 Deletion of ClpX(P) in a  $\Delta$ SocB background produces only full-length DnaX, a protease blind mutant can support growth in a wildtype background**

A. Deletion of either ClpX or ClpP prevents DnaX processing in *Caulobacter*. B. Genetic disruption of the *dnaX* locus that incorporates a protease blind mutant (DnaX<sub>intDD</sub>) is tolerated in *Caulobacter*, and does not generate smaller gamma fragments. C. Wildtype and DnaX<sub>intDD</sub> genotype cells were grown and exposed to DNA damaging agent MMC. Normalized samples were probed for α-DnaX, α-RecA, and α-ClpP. The DnaX<sub>intDD</sub> cell genotype remains stable after DNA damage and exhibits similar RecA induction compared to wildtype cells.

The tau-only background (DnaX<sub>intDD</sub>) was then tested for effects that re-addition of gamma has on this strain. Previously, we have shown that expression of the shortest DnaX fragment, consisting of residues 2-462 (DnaX<sub>ntd</sub>), in conjunction with the not-processed version of DnaX (DnaX<sub>np</sub>) allows for the degradation of the full-length but no longer supports processing. These two versions of DnaX are the minimal requirement for survival when coexpressed in a temperature sensitive background (DnaX<sub>ts</sub>) [2]. We expected that expression of DnaX<sub>ntd</sub> in the



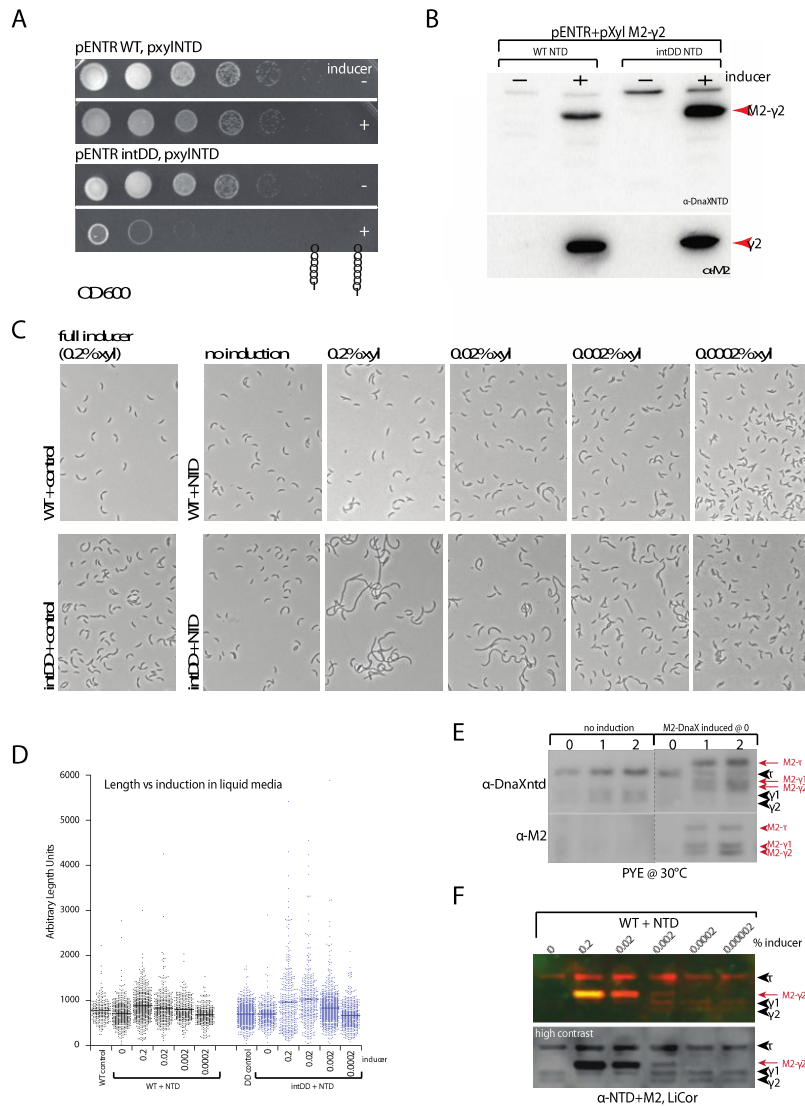
DnaX<sub>intDD</sub> background would have a similar effect on cells as seen before in a wildtype background (DnaX<sub>wt</sub>), since the mutant and wildtype backgrounds otherwise exhibit similar growth, cell-cycle differentiation, and DNA damage response characteristics. This is not an unreasonable expectation as DnaX<sub>intDD</sub> behaves exactly as wild type cells. Remarkably, plasmid expression of DnaX<sub>ntd</sub> in the DnaX<sub>intDD</sub> background inhibits growth (Figure A.4A). In contrast, plasmid expressed M2- DnaX<sub>ntd</sub> in the DnaX<sub>wt</sub> strain remains unaffected by expression extra gamma protein produced *in trans*. Interestingly, expression of M2-DnaX<sub>ntd</sub> at similar induction levels that produces near identical quantities of detected M-2 tagged gamma protein (Figure 4B, anti-M2) also shows more protein under anti-DnaX antibody detection at the size corresponding to M2- DnaX<sub>ntd</sub> (Figure A.4B,  $\alpha$ -DnaX<sub>ntd</sub>). The difference between signal intensity is not readily explained, but a tempting speculation is that the M2-DnaX<sub>ntd</sub> protein allows for positive ClpXP engagement of the DnaX<sub>intDD</sub> protein. This logic potentially extends to further speculation that additional supplied gamma *in trans* increases distal contacts that orient full-length DnaX, increase recognition, and helps ClpX recognize an otherwise not normally degraded DnaX<sub>intDD</sub>.

Overexpression of gamma in the DnaX<sub>intDD</sub> background causes a cell elongation phenotype not seen in when expressed in wildtype or when DnaX<sub>wt</sub> is present (Figure A.4C, quantification in 7.4D). This morphology change directly correlates with loss of cell survival seen via corresponding to similar M2-DnaX<sub>ntd</sub> induction levels on plated growth assays. In this case, expression of gamma in the DnaX<sub>intDD</sub> background is only tolerated under conditions of lower level expression. Interestingly, these lower expression levels of gamma in the DnaX<sub>intDD</sub> background does not cause morphological defects either (Figure A.4C, Figure A.5).

That the *Caulobacter* system supports having a tau-only DnaX without dramatically changing the genome content suggests that the regulatory system charged with maintaining DnaX levels as a whole adapts to the activity of current protein levels. Since the system is already challenged to adjust for tolerating only having the full-length protein, being newly intolerant of

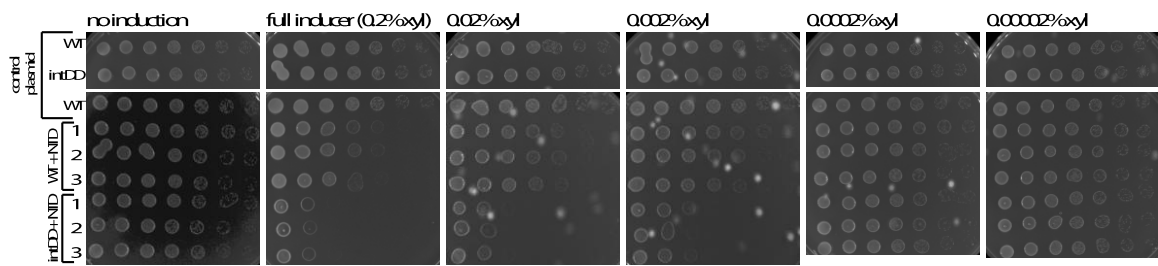
gamma expression may be simply explained through having a sensitized system trying to react beyond its capabilities. Simply, expression of gamma creates more stress on the response system and challenges the initial accommodation for supporting tau-only expression from the *dnaX* locus. Possibly, integration of the gamma in the DnaX<sub>ntd</sub> creates a non-degradable clamp loading system completely resistant to ClpXP based remodeling, a process required for full activity of the loader.

The expression of M2-DnaX<sub>wt</sub> provides negative feedback that suppresses expression of endogenously supplied DnaX levels and the resulting processing (as seen in Figure A.2D, Figure A.4E), validating that expression *in trans* can complement native protein levels and activity. In contrast, overexpression of M2-DnaX<sub>ntd</sub> does not negatively affect or regulate the expression of full-length protein *in vivo*. Expression of M2-DnaX<sub>ntd</sub> does not suppress expression of endogenous full-length DnaX; instead M2-gamma supplied *in trans* influences the amount of endogenous gamma generated from the processing of full-length DnaX (Figure A.4F). The supply of gamma *in trans* only complements the amount of gamma generated from the endogenous full-length, suggesting a stoichiometric maintained balance based on the current existing clamp loader proteins.



**Figure A.4** Gamma expression in DnaX<sub>intDD</sub> background is not tolerated, gamma fragments may autoregulate production

A. Expression of  $\gamma_2$  in the DnaX<sub>intDD</sub> background reduces viability of *Caulobacter* on plated growth. B. Plasmid production of M2- $\gamma_2$  in cells growing in PYE induces similar levels of protein. Interestingly, more protein is visualized at the M2- $\gamma_2$  level when  $\alpha$ -DnaXntd antibodies are used. C. Only DnaX<sub>intDD</sub> cells show a distinct change in morphology upon induction of M2- $\gamma_2$  that dissipates under lower titrative conditions (quantified in D). E. Condensed from Figure A.2D, Induction of M2-DnaX effects total endogenous protein produced and processed. F. M2- $\gamma_2$  protein expressed in a background that produces DnaX<sub>WT</sub> from the endogenous locus specifically changes the amount of endogenous  $\gamma_2$  generated from the processing of DnaX<sub>WT</sub>. M2- $\gamma_2$  does not alter or have feedback effects onto the concentration of DnaX produced, but provides a direct substitute for  $\gamma_2$ .



**Figure A.5 M2- $\gamma$ 2 expression generates a dose dependent lethality in DnaX<sub>intDD</sub> cells**

Overexpression of M2- $\gamma$ 2 from pXYL greatly reduces viability down to three orders of magnitude less inducer. The amount of induced M2-protein produced at viable levels is negligible, and can barely be seen by western (example, Figure A.4F).

**Table A.1 List of mutations that exist in DnaX<sub>intDD</sub> that may allow for tolerating a tau-only DnaX background**

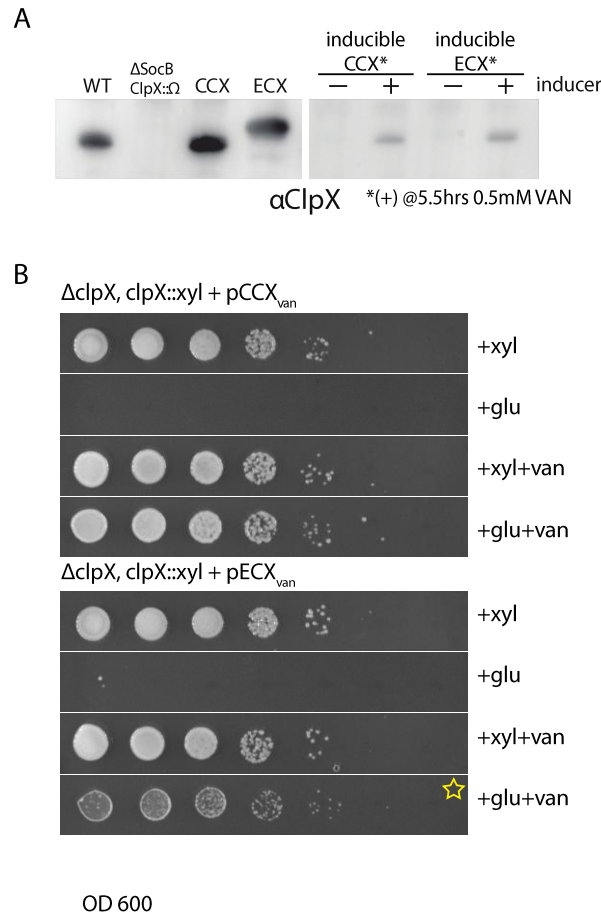
Three biological replicates of the genotype dnaX<sub>intDD</sub> had similar genomic mutations not shared with the progenitor NA1000 (wildtype) strain. Examples highlighted in grey exhibit solidarity between mutants, and those shaded in light blue show mutations that were found in two out of the three mutant replicates. Data between strains was initially analyzed in the breseq format, and each mutation was individually validated against the *Caulobacter* NA1000 reference genome found at ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

Mutation predictions			Location on NA1000 genome				
potential	type	description	WT	DD1	DD2	DD3	LMT
1 good	inversion/repeat	ORF/promoter	none	119115-119196 119217-119143	119115-119196 119217-119152	118971-119052 119057-118983	Either messes with Thioredoxin production, or changes production of Lon endopeptidase-related protein
2 good	inversion/duplication	ORF	none	242723-242641 242673-242747	242723-242641 242673-242747	242723-242641 242673-242747	potentially messes up TonB receptor protein production (Fe <sup>+</sup> transport into the periplasmic space) implicated in hydroxy-radical mediated cell death (HU)
3 good	inversion/duplication	ORF CCNA_00457	none	469266-469336 469440-469359	469485-469411 469438-469518	469266-469336 469440-469359	messes with the end of PEP transferase HPr/PTS system (glucose-specific IIA subunits) messes with energy gained during sugar uptake (phospho relay)
4 not strong	insertion element	ORF CCNA_00615	none	374-388	374-388	374-388	TonB dependent receptor (potentially messing with Ferric chelates)
5 weak	insertion element	ORF CCNA_0660	none	GACG insert 436-440	none	GACG insert 436-440	insert into transposase
6 weak	inversion	ORF CCNA_0660	none	718515-718595 718632-718558	718515-718595 718632-718558	none	inversion in transposase
7 okay	inversion/deletion duplication	ORF CCNA_01264	none	1394867-1394947 1395154-1395081	1395032-1394967 1395125-1395199	1395028-1394947 1394972-1395041	inversion in transposase
8 good	inversion/deletion	ORF CCNA_02828	none	2983420-2983347 2983376-2983461	2983420-2983347 2983376-2983461	2983421-2983347 2983376-2983461	end of transposase (operon) into CCNA_02829. S8 peptidase (stimulates limited proteolysis)
Marginal mutation predictions							
1 weak	inversion/deletion	intergenic CCNA_00682>>CCNA_00683	none	738665-738800	738667-738796	738677-738791	intergenic region downstream of converging ORFs (MarR trans regulator and Deoxyribodipyrimidine photo-lyase-related protein)

### **A.5 *E. coli* ClpX is overactive in *Caulobacter*; reduction in protein level facilitates *Caulobacter* survival**

Previous studies have demonstrated that substitution using *E. coli* ClpX (ECX) does not complement the activity of ClpX in *Caulobacter* [6]. Co-expression of ECX in conjunction with *Caulobacter* ClpX (CCX) show decreased survival of cells plated compared to control (Figure 2A in [5], CPC26 plus pECX), where expression of ECX alone cannot support viability. During co-expression, ECX competes with CCX for ClpP binding, likely impairing ClpP activity by reducing productive CCX-ClpP interactions.

Expressing ECX from the *Caulobacter* ClpX promoter fails to support robust viability. Interestingly, expression of ECX from a lower copy plasmid that reduces protein output upon induction yields different results (Figure A.6A). Using a vanillate inducible plasmid that produced lower levels of the ECX protein promotes plated *Caulobacter* growth. The growth was not as robust as a wildtype repletion under similar conditions; however, both strains grew out to the similar CFU dilutions via plated media (Figure A.6B, *star*). This data suggests that lower concentrations of the ECX protein minimally substitutes in complementing the required ClpX degradation activity. ECX protein production at similar levels to endogenous CCX levels is simply not tolerated (Figure 1A, ClpX levels from  $\Delta$ ClpX,  $\Delta$ SocB strain, compared to ClpX expressed from  $P_{xyI}$  *clpX* induction with empty plasmid in Figure 2B of [5]). These data suggest that ECX activity in *Caulobacter* is unregulated, with much higher or off-target activity, and thus detrimental to *Caulobacter* survival. Expressing much less ECX protein in *Caulobacter* barely supports viability. Excessive amounts of ECX in *Caulobacter* fail to support survival. ECX poorly support the ClpX system in *Caulobacter*, which requires the fine-tuning and layers of regulation on ClpX that ensures normal growth.



**Figure A.6 Having some *E. coli* ClpX is better than none**

A. Use of the *Caulobacter* clpX promoter on plasmid produces physiological ClpX concentrations of both *Caulobacter crescentus* (CCX) and *E. coli* (ECX) ClpX. Inducing these proteins from a vanillate (VAN) promoter produces much less ClpX protein. B. (Each section taken from separate plates that contain the indicated inducers) Maintaining CCX levels in a ClpX depletable strain (+xyl) promotes *Caulobacter* growth, where the absence (no xyl, +glu) does not. Co-expression of CCX and lower concentrations of CCX and ECX do not negatively impact growth (+xyl+van). Lower levels of ECX does support *Caulobacter* viability to similar plated dilutions, however, growth does not look robust when compared to when CCX is present.

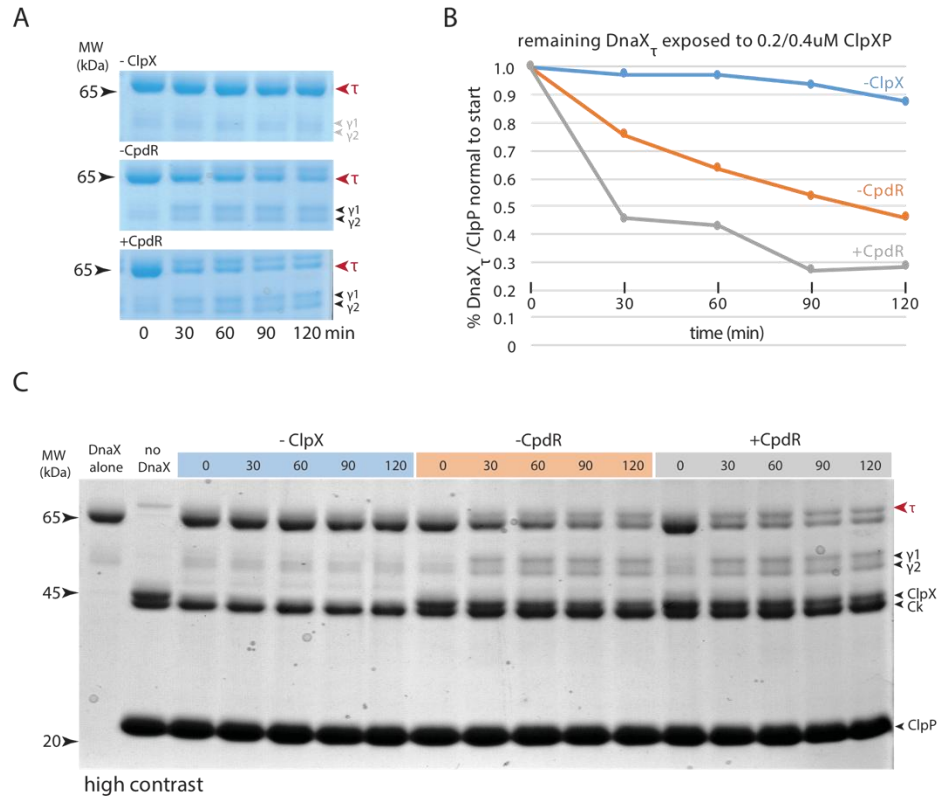
#### **A.6 CpdR may modulate the speed at which DnaX is processed.**

The unfoldase ClpX delivers substrates to ClpP for destruction and uses additional specificity factors called adaptors that gate activity by modulating interactivity. Adaptors modulate activity by changing substrate interactions with the unfoldase therefore increasing the effective substrate pools. Adaptors of ClpXP have several modes in which they change interaction, and are classified by their action with substrate delivery. Substrate delivery can be dependent, enhanced or inhibited by that adaptor [7]. Our studies show that adaptor CpdR specifically mediates proteolysis directly for the substrate PdeA [8], yet CpdR addition to *in vitro* assays enhances degradation of substrates already recognized and degraded by ClpXP (specifically DnaX). For example, CpdR drives the degradation of CtrA *in vitro* when the full adaptor hub assembly is available [9].

Partial processing of DnaX is not dependent on the presence of CpdR (Figure A.7). Further *in vitro* analysis suggests that addition of CpdR does not increase the formation of gamma from full-length DnaX when both the protease and adaptor are present (Figure A.7A,C). The addition of CpdR does not change the proteolytic output of ClpXP, nor does it change the proportion of gamma generated from the full-length DnaX protein, suggesting addition of CpdR increases the degradation rate of DnaX (Figure A.7B). This leads to a speculation that CpdR increases ClpXP activity by increasing the sensitivity towards normally recognized protein targets. Additionally, the adaptor CpdR may possess alternative activity from its known role of providing a scaffold base for the adaptor-hub assembly and directly mediating PdeA degradation. Perhaps CpdR simply supercharges the ClpX unfoldase to recognize its protein substrates in a more efficient manner. This would offer an advantage to tune a processing response of DnaX recognition through a response regulator, or during low energy availability. Perhaps CpdR-based changes in DnaX processing balances processing as the DnaX protein is needed, especially during



DNA damage and cell differentiation. Changes in processing speed could effectively alter ClpXP activity to counteract increases in substrate load.



**Figure A.7 CpdR speeds up the degradation of DnaX<sub>FL</sub> but does not affect processing**

A. *In vitro* degradation and processing of DnaX into gamma requires ClpX. The addition of CpdR (2.5μM to 0.2/0.4μM XP) increases the degradation rate of full-length DnaX (DnaX<sub>FL</sub>). B. Quantified loss of DnaX<sub>FL</sub> reveals that the addition of ClpX adaptor CpdR increases the initial rate and extent that DnaX<sub>FL</sub> is degraded given the same amount of time. C. Side by side analyses with loading controls. DnaX<sub>FL</sub> degradation and gamma generated by ClpXP processing. Graph in section B was normalized to ClpP levels.

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